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Determination of Benzidines in Industrial and Municipal Wastewaters

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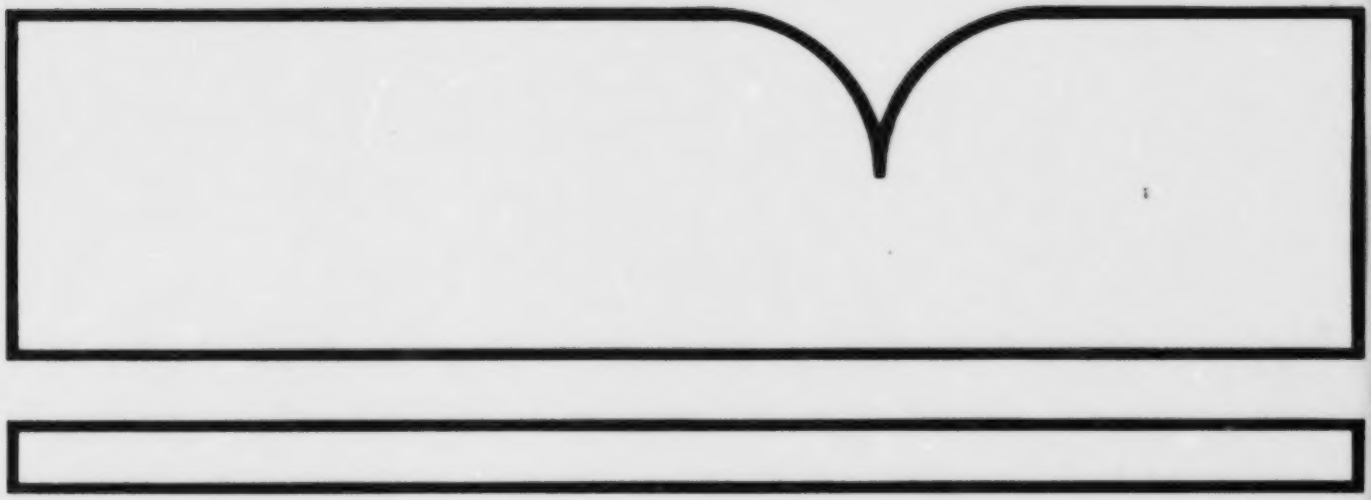

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April 1982

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DETERMINATION OF BENZIDINES IN INDUSTRIAL AND MUNICIPAL WASTEWATERS

by

Ralph M. Riggin and C. C. Howard
Battelle Columbus Laboratories
Columbus, Ohio 43201

Contract No. 68-03-2624

Project Officer

James E. Longbottom
Physical and Chemical Methods Branch
Environmental Monitoring and Support Laboratory
Environmental Protection Agency
Cincinnati, Ohio 45268

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16. ABSTRACT

A method for the determination of benzidine and 3,3'-dichlorobenzidine in wastewater was developed. This method, based on the use of high performance liquid chromatography with electrochemical detection, is selective for readily oxidized, basic compounds, and can detect as little as 0.1 ppb of benzidine and DCB in many types of aqueous samples, including surface water, municipal sewage, and industrial effluent samples. However, several dye plant effluents contained many oxidizable materials which decreased the sensitivity of the method to 1 ppb for benzidine and DCB. Apparent levels of 10-20 ppb of benzidine were present in the dye plant effluents.

Precision and accuracy of the method were estimated from the results for five wastewater samples spiked at levels between 1 and 50 ppb. For this group of samples the recoveries were 69 ± 15% for benzidine and 76 ± 9% for DCB.

Storage of several wastewater samples for (2 or 7 days) at 4°C and pH 2 resulted in degradation of the compounds in several cases, probably due to oxidation or irreversible adsorption to particulate matter. Therefore, in order to obtain accurate values for benzidine and DCB it is believed to be necessary to assay the sample as soon after collection as possible. For samples containing chlorine, a reducing agent such as sodium thiosulfate must be added since chlorine was found to rapidly degrade benzidine and DCB.

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FOREWORD

Environmental measurements are required to determine the quality of ambient waters and the character of waste effluents. The Environmental Monitoring and Support Laboratory - Cincinnati conducts research to:

- Develop and evaluate techniques to measure the presence and concentration of physical, chemical, and radiological pollutants in water, wastewater, bottom sediments, and solid waste.
- Investigate methods for the concentration, recovery, and identification of viruses, bacteria, and other microbiological organisms in water. Conduct studies to determine the responses of aquatic organisms to water quality.
- Conduct an Agency-wide quality assurance program to assure standardization and quality control of systems for monitoring water and wastewater.

Under provisions of the Clean Water Act, the Environmental Protection Agency is required to promulgate guidelines establishing test procedures for the analysis of pollutants. The Clean Water Act Amendments of 1977 emphasize the control of toxic pollutants and declare the 65 priority pollutants and classes of pollutants to be toxic under Section 307(a) of the Act. This report is one of a series that investigates the analytical behavior of selected priority pollutants and suggests a suitable test procedure for their measurement.

Robert L. Booth

Robert L. Booth, Acting Director
Environmental Monitoring and Support
Laboratory - Cincinnati

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ABSTRACT

A method for the determination of benzidine and 3,3'-dichlorobenzidine in wastewater was developed. This method, based on the use of high performance liquid chromatography with electrochemical detection, is selective for readily oxidized, basic compounds, and can detect as little as 0.1 ppb of benzidine and DCB in many types of aqueous samples, including surface water, municipal sewage, and industrial effluent samples. However, several dye plant effluents contained many oxidizable materials which decreased the sensitivity of the method to about 1 ppb for benzidine and DCB. Apparent levels of 10-20 ppb of benzidine were present in the dye plant effluents. For one dye plant effluent benzidine was determined to be 9 ppb and 12 ppb using two different sets of chromatographic conditions, thus supporting the belief that benzidine is present at the level stated.

Precision and accuracy of the method were estimated from the results for five wastewater samples spiked at levels between 1 and 50 ppb. For this group of samples the recoveries were 69 ± 15% for benzidine and 76 ± 9% for DCB.

Storage of several wastewater samples for (2 or 7 days) at 4°C and pH 2 resulted in degradation of the compounds in several cases, probably due to oxidation or irreversible adsorption to particulate matter. Therefore, in order to obtain accurate values for benzidine and DCB, it is believed to be necessary to assay the sample as soon after collection as possible. For samples containing chlorine, a reducing agent such as sodium thiosulfate must be added, since chlorine was found to rapidly degrade benzidine and DCB.

This report was submitted in fulfillment of Contract No. 68-03-2624 by Battelle Columbus Laboratories under the sponsorship of the U. S. Environmental Protection Agency. This report covers a period from November 1, 1977, to March 1, 1979, and work was completed as of March 1, 1979.

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SECTION 1

INTRODUCTION

This report presents the results obtained under Contract No. 68-03-2624, in which a method was developed for the determination of benzidine, dichlorobenzidine, and diphenylhydrazine in aqueous effluents. This work was conducted in two phases, wherein Phase I consisted of evaluation of various analytical methodologies, and Phase II consisted of validating the most promising method using several aqueous effluent samples. Data from the Phase I study were presented in a report dated May 19, 1978, and are also presented herein.

The objective of this study was to develop an optimized analytical method for the determination of benzidine, dichlorobenzidine (DCB) and 1,2-diphenylhydrazine (DPH) and to validate the method on a variety of aqueous effluents.

The successful completion of this program involved the fulfillment of certain directives set forth in the contract by EPA. An extensive literature review was first conducted to evaluate the previous work in the area. Subsequent work was directed toward determination and then full evaluation of an appropriate measurement technique, which best satisfied the requirements for sensitivity and selectivity, as well as the considerations of sample cost, i.e. equipment, time, and training, which would be needed for the method. The stability of the benzidine compounds in water miscible solvents and their instability in chlorinated and unchlorinated buffered water at different pHs and storage temperatures were studied over the prescribed time periods. Extraction efficiency of two organic solvents was also studied for the standard compounds. The remainder of the program involved the study of the sample preparation and clean-up steps which would be necessary to eliminate sample interferences. The complete method was then applied to several representative wastewater samples and an assessment was made of the precision and accuracy of the complete procedure.

SECTION 2

LITERATURE REVIEW

Preliminary efforts relied totally on the use of automated searches of Chemical Abstracts, Water Resources Abstracts, and National Technical Information Service. While these searches found a few interesting papers, it was apparent that a manual search of Chemical Abstracts and Analytical Abstracts needed to be conducted, and this was done.

Preliminary analytical methods for benzidines have been mainly colorimetric with only a few studies using gas chromatography. The chloranil reaction is used extensively but is not highly specific.[1] A recent paper compares the use of colorimetry, thin layer chromatography, and gas chromatography (GC) for the determination of benzidine in wastewaters.[2] GC was found, not surprisingly, to be the method of choice. However, only the FID was used and a detection limit of 100 ppb injected was attained. No literature has been found where alkali flame (AFD) or electron capture detection (ECD) was employed. One study employed spectrophotofluorimetry for benzidine analysis in wastewater and several other matrices with a detection limit of 10 ppb.[3]

Recent studies using HPLC with electrochemical detection (HPLC/EC) have been very encouraging.[4] A comparison of HPLC/EC with GC/AFD was made and HPLC/EC was found to be at least 100 times more sensitive.

Conclusions based on the literature survey are as follows:

(1) GC methods have not been evaluated sufficiently, in that several possible modes of detection have been explored (at least not that we know from the literatuare searches made).

(2) HPLC with electrochemical detection is definitely a prime candidate for the method of choice and should be evaluated further.

(3) The fluorimetric behavior of benzidine suggests HPLC/fluorescence might work but the limits of detection probably would not be as good as HPLC/EC.

SECTION 3

EXPERIMENTAL PROCEDURES

ANALYTICAL METHODS DEVELOPMENT

Three analytical techniques were evaluated for potential use in the determination of benzidines: (1) direct GC/AFD, (2) GC/ECD following derivatization with a fluoroacyl group; and (3) HPLC/EC.

Direct GLC/AFD

Gas chromatographic properties of the three compounds were investigated using a Hewlett Packard 5730 gas chromatograph equipped with dual FID/AFD. Columns were 6 ft x 2 mm I.D. glass, packed with a 3% loading of the liquid stationary phase on Gas Chrom Q 100/120. The stationary phases evaluated were OV-1, OV-17, SP2250DB, and OV-225 which represent a wide range of polarities. The gas chromatographic conditions were as follows:

Temperature program - 140° to 240° at 8°/min
Flow rate - 30 mL/min Helium

GC/FID was used to evaluate all four stationary phases and GC/AFD was evaluated for the SP2250DB stationary phase. The linearity of response for the three compounds was determined by injecting 2 µL quantities of standard solutions in methanol at appropriate concentrations. Quantitation was based on the peak areas obtained from triplicate injection using a Spectra Physics Model 4000 data system.

During this investigation, it was noted that DPH sometimes gave more than one peak, and the larger of the peaks eluted much earlier than expected. In order to determine the identity of the components eluted, combined gas chromatography/mass spectrometry (GC-MS) was employed. Two microliters of a 50 ng/µL solution of DPH in toluene was injected onto the GC-MS system using the following conditions:

Instrument -- Finnegan 3200
Injector temperature -- 200°C
Injection -- splitless at 80°C
Column -- 30 meter SE-30 glass capillary
Flow rate -- 2 mL/min
Injection volume -- 2 microliters
Temperature -- 80-260°C at 10°C/min
Ionization -- EI at 70 eV

Multiplier voltage -- 2400 V
Sensitivity -- 10^{-7} Amps/Volts
Scan mode -- IFSS
Integration time -- 5 MSEC
Scan range -- 40-350 AMU

GC/ECD

The use of various derivatization reagents to form highly electron capturing derivatives was investigated. The following derivitization reagents were evaluated: (1) trifluoroacetic anhydride (TFA); (2) pentafluoropropionyl anhydride (PFPA); (3) heptafluorobutyric anhydride (HFBA); (4) trifluoroacetyl imidazole (TFI); and (5) heptafluorobutryl imidazole (HPBA). In all cases, the following GLC parameters were used:

GC -- HP5730A
Column -- 3% OV-17 on Supelcoport 100/120
6 ft x 2 mm I.D. glass
Flow rate -- 30 mL/min Helium
Temperature program -- 100°-300° at 8°C/min
(held at 100° two minutes after injection)
Detector -- 300°C (FID)
Injector -- 200°C
Attenuation -- 1 x 32
Injection Volume -- 2 microliters

Amines were reacted individually with each of the derivatizing agents using reaction conditions described in the Pierce Chemical catalogue.

Anhydrides--
One hundred micrograms of the amine in 100 microliters of benzene were mixed with 10 microliters of the anhydride, 50 microliters of 0.5M triethylamine, and 300 microliters of benzene in a 15 mL screw capped centrifuge tube. The mixture was heated at 50°C for 20 minutes and then allowed to cool to room temperature. One milliliter of distilled water was then added and the mixture shaken for one minute. One milliliter of 5% ammonia was then added and shaken for five minutes. The mixture was centrifuged and the benzene layer placed in a GC vial for analysis.

Imidazoles--
One hundred micrograms of the amine in 100 microliters of benzene was mixed with 200 microliters of the imidazole and heated at 60°C for 30 minutes. The mixture was injected directly onto the gas chromatograph.

HPLC With Electrochemical Detection

This system was constructed from modular components using readily available equipment as follows:

Solvent delivery system -- Altex model 110A
Injector -- Water's model U6K

Detector -- Bioanalytical systems model LC-2A
Electrode -- Glassy carbon (3 mm diameter)
Injection volume -- 25 microliters
Output device -- Strip Chart recorder 0-10V full scale

In order to determine the optimum conditions for detection of these compounds, several mobile phase compositions, stationary phases, and electrode potentials were investigated. Mobile phases consisted of various buffers including acetate and citrate/phosphate at appropriate concentrations and pH values combined with either methanol or acetonitrile. All solvents were filtered through a Millipore .22 µM filter prior to use on the HPLC.

Stationary phases evaluated include:

1. Zorbax ODS
2. Zorbax CN
3. µ Bondapak C-18
4. Whatman Partisil - SCX
5. Lichrosorb RP-18
6. Lichrosorb RP-8
7. Lichrosorb RP-2
8. Spherisorb ODS

All columns were 25 cm x 4.6 mm I.D., stainless steel, except the µ-Bondapak C-18 which was 30 cm x 4.6 mm I.D. The first four columns were purchased commercially, whereas the latter four were slurry packed using conventional techniques.[5]

In order to determine the optimum electrode potential to use, a series of injections were made of a 1 ppm solution the three compounds in .1M sodium acetate buffer (pH 4.7) at various electrode potentials from .4-.9V vs (Ag/AgCl). The linearity of the chromatographic response was determined by injecting 25 µL volumes of the compounds contained in a .1M sodium acetate buffer (pH 4.7) using an electrode potential of 0.8 volts versus Ag/AgCl. Response was determined by measuring the peak height for each component.

SOLVENT STABILITY STUDIES

The stability of the three compounds in water miscible solvents was studied over a 90 day period. In this study, benzidine and DCB were stored separately from DPH, since DPH can be degraded to benzidine.

Four milliliter quantities of 100 ppm solutions of the compounds were flame sealed in 20 milliliter glass ampules and stored in the dark at room temperature. DCB and benzidine were stored in acetonitrile and methanol whereas DPH was stored in a number of different solvents, including benzene, methylene chloride, methanol, acetonitrile, triethylamine, and acetic acid since it was found to have poor storage stability in our initial studies.

At 0, 30, 60, and 90 days after storage, three ampules were opened and

analyzed. A 100 microliter aliquot of the solution was diluted to 10 milliliters with .1M acetate buffer (pH 4.7), well shaken, and analyzed by HPLC/EC, using the following conditions:

Solvent delivery system -- Altex model 110A
Injector -- Water's model U6K
Column -- Lichrosorb RP-2, 4.6 mm I.D. x 25 cm long, 5 micron particle diameter
Mobile phase -- 50% acetonitrile, 50% .1M sodium acetate buffer (pH 4.7)
Flow rate -- 0.8 mL/min
Detector -- Electrochemical, Bioanalytical Systems model LC2 equipped with glassy carbon electrode
Detector voltage -- 0.85 volts versus Ag/AgCl
Injection volume -- 25 microliters

Quantitation was achieved by comparison of the peak heights obtained to those of freshly prepared standards. The data obtained was subjected to a oneway ANOVA analysis using the Statistical Package for the Social Sciences (SPSS) program.

EXTRACTION STUDIES

The extractability of the three compounds from aqueous solution at various pH values was investigated. Both methylene chloride and chloroform preserved with 2% ethanol were evaluated as extraction solvents.

The procedure employed was as follows:

1. Five hundred milliliters of the appropriate buffer solution were spiked with 50 microliters of a 100 ppm solution of the three compounds to give a level of 10 ppb. Separate extractions were performed for benzidine and DPH, due to the possible degradation of DPH to benzidine. Buffers used were .1M Na_2HPO_3 at pH 7, .1M sodium borate at pH 10, and 1M citric acid at pH 2 (exact pH values were obtained by adding 1M H_2SO_4 or 1M NaOH).

2. The aqueous solution was extracted with 50 and then 30 milliliters of organic solvent in a 1 liter separatory funnel, and the organic extracts were combined and washed with 20 milliliters of water in a 250 milliliter separatory funnel.

3. The organic extract was placed in a 100 milliliter round bottom flask with 20 milliliters of methanol and concentrated to about 5 milliliters on a rotating evaporator at 35°C.

4. The concentrate was then transferred to a 15 milliliter conical screw capped centrifuge tube using a Pasteur pipette. The flask was rinsed with 2 milliliters of methanol which was added to the centrifuge tube.

5. The sample was then concentrated to 2 milliliters on a vortex evaporator (Buchler Model 3-2200) at 35°C, diluted to 4 milliliters with .1M sodium acetate buffer at pH 4.7, and analyzed by HPLC with electrochemical detection using the conditions described in the Solvent Stability Section.

Triplicate extractions were performed using each organic solvent at each pH level. The data obtained was subjected to ANOVA analysis.

In addition to these studies, a study was undertaken to determine which of several different concentration techniques were best for the benzidines. Two micrograms of benzidine and DCB were dissolved in 70:30 chloroform/methanol and concentrated in one of the following ways:

1. Vortex evaporation -- The solution was placed in a 15 mL conical centrifuge cube and concentrated to .5 mL using a vortex evaporator at 30°C,

2. Nitrogen evaporation -- The solution was placed in a 15 mL conical centrifuge tube and concentrated to .5 mL by blowing a gentle stream of nitrogen into the tube which was immersed in a 30° water bath. Two mL methanol were added and the sample reconcentrated to .5 mL.

3. Kuderna-Danish (K-D) -- The solution was placed in a 25 mL Kontes K-D tube fitted with a modified micro Snyder column. The sample was concentrated to about .5 mL either by heating with a tube heater (modified to heat only the tip of the tube) at 100°C or by immersion in a water bath at 60°C. The joint of the K-D tube and Snyder column was rinsed with 1 mL methanol.

Each of the concentrates was diluted to 5 mL with .1M acetate buffer, pH 4.7 and analyzed by HPLC/EC. Each concentration mode was run in triplicate.

A second experiment was run to test the recovery of benzidine using the protocol currently being used for surveying wastewater effluents. Five hundred milliliters of distilled water were spiked at the 10 ppb level with benzidine and DCB. This solution was then extracted and concentrated using the procedure described for base/neutral extractables in "Sampling and Analysis Procedures for Screening of Industrial Effluents for Priority Pollutants," EPA (EMSL-Cincinnati), April 1977. The concentrate (methylene chloride) was displaced with methanol by vortex evaporation and analyzed by HPLC/EC.

STORAGE STABILITY STUDIES

The stability of the benzidines in water under a variety of storage conditions was investigated. Two temperatures, 4°C and room temperature, three pH levels, 2, 7, and 10, and two chlorine levels, 0 and 2 ppm, were evaluated by preparing duplicate 500 milliliter samples spiked with 10 ppb of DCB and benzidine or DPH. DPH was done separately since it can degrade to benzidine under certain storage conditions. The solutions were stored in amber glass bottles and stored for seven days prior to extraction, using

methods described in the previous section. Before extraction, the pH was adjusted to pH 7 with 1M H_2SO_4 or 1M NaOH.

The pH values were achieved using the same buffer concentrations as were used for the extraction studies. The 2 ppm chlorine level was achieved by addition of two milliliters of a 1500 ppm aqueous solution of calcium hypochlorite to 500 milliliters of water. These variables defined a 2x2x3 matrix of conditions and each condition was run in duplicate to give a total of 24 analyses. The data obtained was then subjected to ANOVA analysis.

Since DPH was found to be highly unstable, it was considered useful to determine its half-life in a representative aqueous medium. Ten milliliters of a secondary sewage effluent were spiked with 100 ppb DPH and the solution was assayed by direct injection of a 25 mL aliquot onto the HPLC/EC system at various time intervals. This experiment was repeated with the sample being purged with nitrogen before spiking and during analysis.

WASTEWATER ANALYSIS STUDIES

Based on extraction efficiency studies, the following procedure was initially employed for the analysis of actual wastewater samples.

Spike sample with 10 ppb benzidine and dichlorobenzidine and adjust to pH 7 with 1M H_2SO_4 or NaOH.

1. Transfer sample to a 500 mL separatory funnel, add 50 mL chloroform, shake for one minute, and allow phases to separate.

2. Transfer bottom layer into a second 500 mL separatory funnel and extract the aqueous phase twice more with 30 mL volumes of chloroform.

3. Add 15 mL water to the combined organic extracts and extract, discarding the aqueous or top layer.

4. Place organic layer in a 250 mL round bottom flask, add 15 mL methanol and concentrate to about 5 mL on a rotating evaporator.

5. Transfer the extract to a 15 mL conical centrifuge tube using a Pasteur pipette. Rinse flask with 2 mL methanol and combine the original extract.

6. Concentrate to about .2-.3 milliliters on a vortex evaporator (evacuated using a water aspirator) at 40°C or with a nitrogen evaporator at 30°C.

7. Dilute sample to 1 mL with chloroform.

8. Extract with 2 x 1 mL 1M H_2SO_4 and centrifuge to separate layers if necessary.

9. Place combined aqueous extracts in a second 15 mL centrifuge tube,

add 3 mL .4M Na_3PO_4, and adjust to pH 7-8 with 1M NaOH, using dropwise addition and thorough shaking. Monitor with pH paper.

10. Extract with 3 x 2 mL chloroform, using a Pasteur pipette to remove organic or bottom layer.

11. Place the combined organic extracts in another 15 mL centrifuge tube, add 2 mL methanol, and concentrate to about .3-.5 mL on the vortex evaporator.

12. Dilute to exactly 2 mL with acetate buffer, mix thoroughly, and assay by injecting 25 microliters into the HPLC.

Since DPH was found to degrade rapidly, even in organic solvents, it was not investigated in this phase of the study.

Quantitation was performed by comparing the peak heights for benzidine and DCB to those of a standard made up to the concentration representing 100% recovery.

Following discussions with EPA personnel, the extraction procedure was modified so that all extractions were performed in a separatory funnel rather than in centrifuge tubes. The revised procedures are described in detail in the Appendix.

The following water samples were selected for analysis:

1. Surface water (Olentangy River)
2. Secondary sewage effluent (Columbus, Ohio)
3. Primary sewage (Columbus, Ohio)
4. Final effluent from plant producing various organic chemicals (such as nitrobenzene, nitrophenols, o-dichlorobenzene, and chloroanilines).
5. Final effluent from plant producing benzidine based dyes (No. 61).
6. Same as above (No. 62).
7. Same as above (No. 63).
8. Oxidation process stream from a plant producing benzidine based dyes.

Each wastewater was analyzed, unspiked in triplicate. Six 500 mL aliquots of each sample were spiked with benzidine and DCB at a level at least five times background. Three aliquots were analyzed immediately and three were analyzed after storage, at pH 2 and 4°C, for a period of time -- either two or seven days. The pH 2 level was attained by addition of 1M H_2SO_4. One gram per liter of sodium thiosulfate was added to each sample prior to spiking.

Although the major thrust of this work was to develop a procedure using solvent extraction techniques, two other methods of analysis were investigated in a preliminary way. The first method involved simply filtering the sample through a 0.45 µM filter (Millipore type GS) and injecting a 25 µL aliquot onto the HPLC/EC system. A single sample from an organic chemicals plant was analyzed using this procedure.

Another concentration technique involved the use of resin adsorption. Ten milliliters of the aqueous sample were passed through a reversed phase cartridge (Sep-pak C-18, Water's Associates) at a flow rate of approximately 10 mL/minute using a glass syringe. The cartridge was then washed with 5 mL distilled water, which was discarded, and eluted with 3 mL methanol. The methanol was then concentrated to 0.5 mL on a vortex evaporator at 30°C, 1 mL of .1M acetate buffer (pH 4.7) was added, and the sample was reconcentrated to 1 mL. This extract was then assayed by HPLC/EC. A secondary sewage effluent was assayed using this approach, before and after spiking with 10 ppb of benzidine and DCB.

SECTION 4

RESULTS

ANALYTICAL METHODS EVALUATION

GC/AFD

The four stationary phases investigated (OV-1, OV-17, SP2250DB, and OV-225) represent a wide range of polarities. Both OV-17 and SP2250DB were found to give satisfactory results. OV-1 was acceptable but some degree of tailing was noted, especially at low concentrations. OV-225 gave an unacceptable degree of column bleed. Chromatograms on OV-1, OV-17, and OV-225 are shown in Figures 1, 2, and 3, respectively. A tabulation of the retention times for each compound on the various columns is given in Table 1.

The use of AFD for the detection of the benzidines was investigated using SP2250DB. Figure 4 shows a separation of 10 nanograms of each of the three compounds using the conditions listed in the experimental section (employing AFD detection). By operating isothermally at 230°C, somewhat better sensitivity (about 1 ng on column) can be achieved for benzidine and DCB; but under these conditions, the DPH peak appears in the solvent front.

Precision of integration areas using the prescribed conditions was ±6 percent for benzidine and ±4 percent for DPH and dichlorobenzidine at the 10 nanogram level (for six replicate 2 µL injections). The detection limit is approximately 2 nanograms for benzidine and dichlorobenzidine and 0.2 nanograms for DPH (as azobenzene) at a signal to noise ratio of 5:1. The linearity of the AFD response for each of the three components is shown in Figure 5.

It should be noted in Figures 1 through 3 that two peaks arise from the injection of DPH, and furthermore, that the larger of the peaks elutes much earlier than would be expected for a reasonably polar compound of this molecular weight. In order to confirm the identity of the eluted components, GC-MS was used as described in the experimental section.

A single sharp peak was observed eluting at 210° (13 minutes) as shown in Figure 6. The mass spectrum revealed (Figure 7) it to be a component with a molecular ion at 182 amu, whereas DPH has a molecular ion at 184. A mass spectrum obtained by solid probe introduction of pure DPH into the mass spectrometer is shown in Figure 8. The component resulting from DPH injection was suspected to be azobenzene (M.W. 182) which was confirmed by injection of pure azobenzene, whose mass spectrum (from GC injection) is

TABLE 1. RETENTION TIMES FOR BENZIDINES ON VARIOUS GAS CHROMATOGRAPHIC STATIONARY PHASES

Column	Retention time (minutes)*		
	Benzidine	DCB	DPH**
OV-1	9.9	10.6	2.1
OV-17	14.5	21.0	6.1
OV-225	17.6	26.5	5.1
SP-2250DB	9.1	14.5	2.1

* Conditions as follows:

Column - 7 feet x 2 mm I.D., glass, .3% loading on Gas Chrom Q 100/120

Temperature program - 140°-240°C at 8°/minute

Flow rate - 30 mL/minute helium

** Largest peak reported

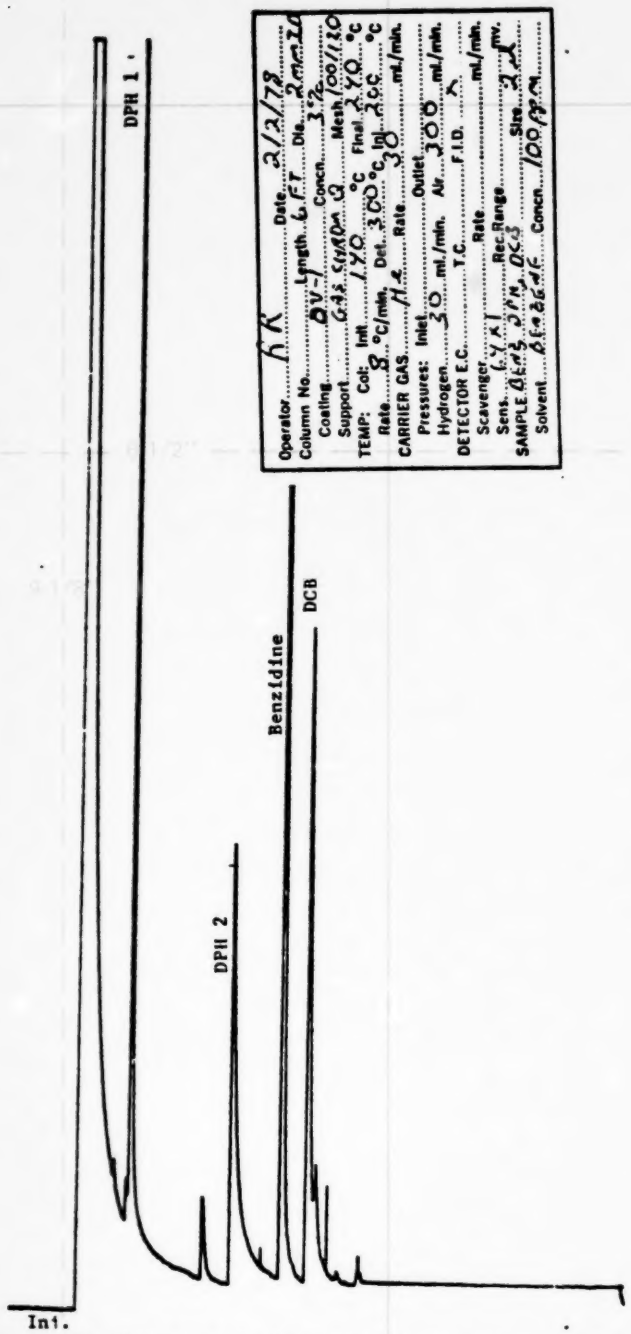

Operator HR Date 2/3/78
Column No. Length 6 F.T. Dia. 2 mm ID
Coating OV-1 Concn. 3%
Support Gas Chrom Q Mesh 100/110
TEMP: Col: Init. 140 °C Final 240 °C °C
Rate 8 °C/min. Det. 300 °C Inj. 250 °C
CARRIER GAS N2 Rate 30 ml/min.
Pressures: Inlet 30 ml/min. Outlet
Hydrogen 30 ml/min. Air 300 ml/min.
DETECTOR E.C. T.C. F.I.D. X
Scavenger Rate ml/min.
Sens. 8 x 10^-12 amps Rec. Range Slw. 2 mv.
SAMPLE Benzidines Solvent Benzene Conc. 100 ppm
DPH 1
DPH 2
Benzidine
DCB
Init.

Figure 1. Gas chromatogram of benzidines using FID on OV-1 temperature programmed from 140° to 240° at 8°/min.

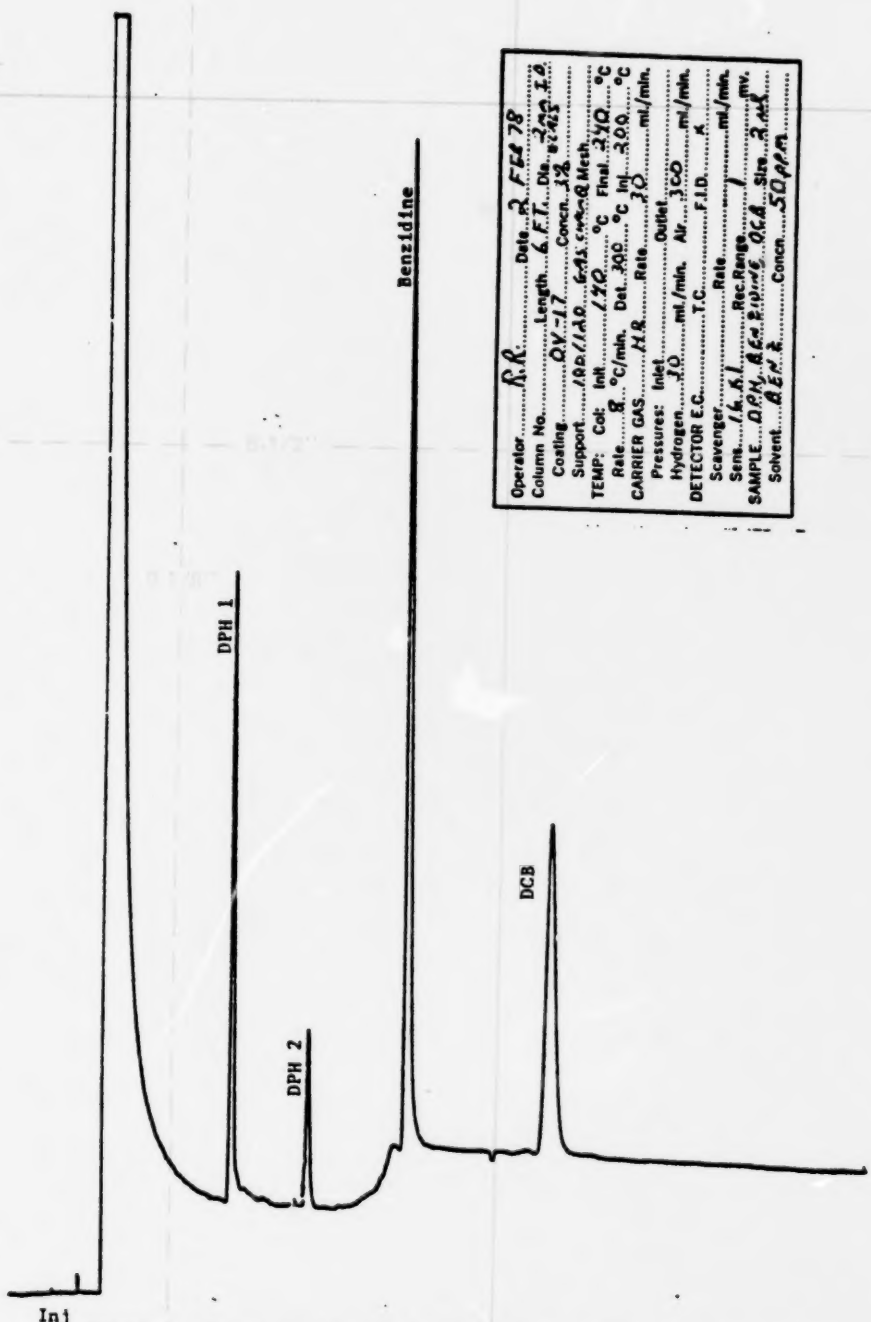
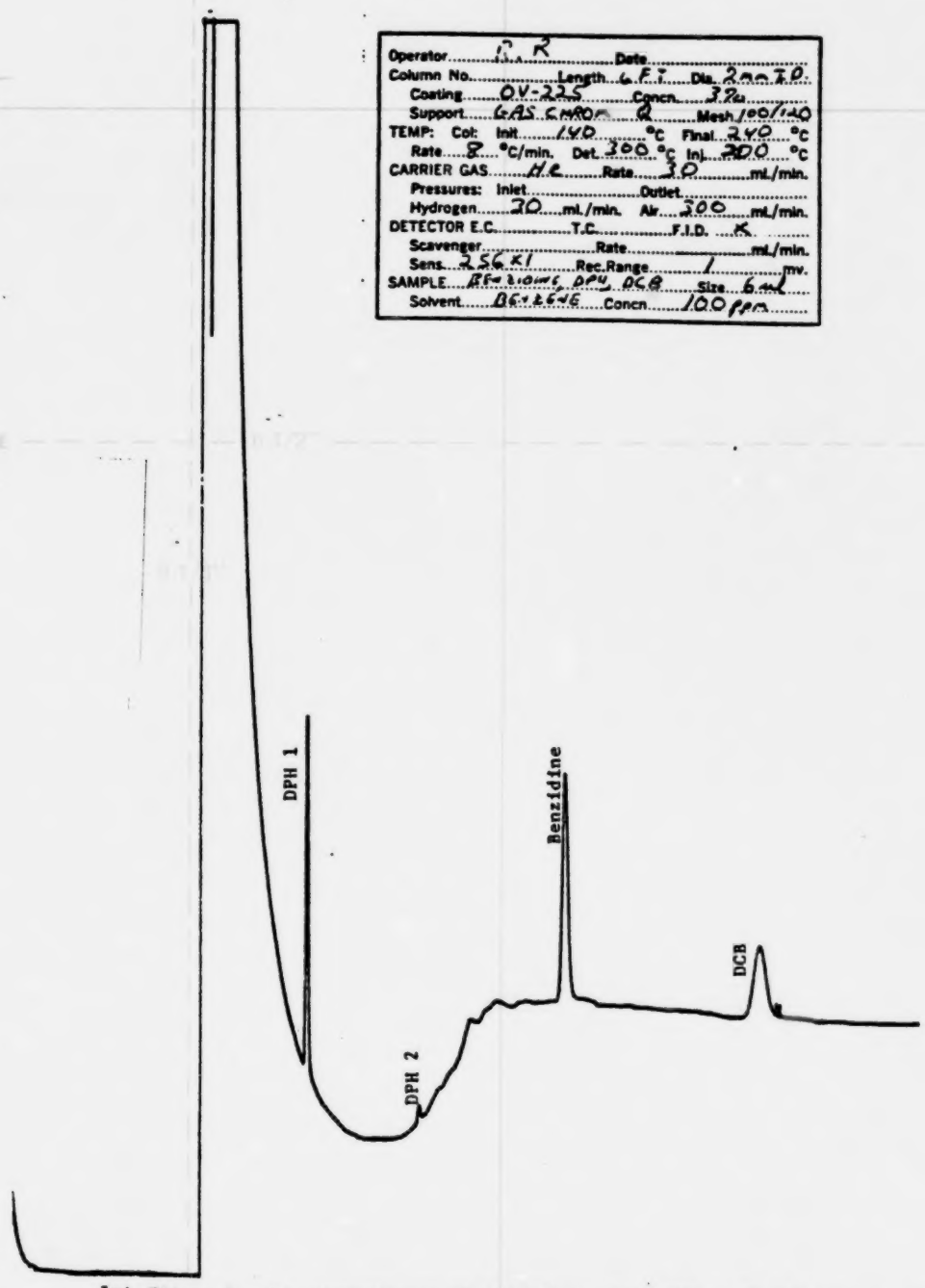

Operator R.R. Date 2 FEB 78
Column No. Length 6 F.T. Dia. 2mm I.D.
Coating OV-17 Concn. 1%
Support 100/120 Gas Chrom Q Mesh
TEMP: Col: Init. 140 °C Final 240 °C
Rate 8 °C/min. Det. 300 °C Inj. 300 °C
CARRIER GAS H.R. Rate 30 ml/min.
Pressures: Inlet Outlet
Hydrogen 10 ml/min. Air 300 ml/min.
DETECTOR E.C. T.C. F.I.D. A
Scavenger Rate ml/min.
Sens. 1x10-1 Rec.Range 1 mv.
SAMPLE O.P.V. Acetylated DCB Sim. 2 µl
Solvent Acetone Concn. 50 p.p.m.
Benzidine
DPH 1
DPH 2
DCB
Inj

Figure 2. Gas chromatogram of benzidine using FID on OV-17 temperature programmed from 140° to 240° at 8°/min.


Operator T. R Date
Column No. Length 6 F. Dia. 2mm ID.
Coating OV-225 Concn. 3%
Support GAS CHROM Q Mesh 100/120
TEMP: Col: Init 140 °C Final 240 °C
Rate 8 °C/min. Det. 300 °C Inj. 200 °C
CARRIER GAS H.E. Rate 30 ml./min.
Pressures: Inlet Outlet
Hydrogen 30 ml./min. Air 300 ml./min.
DETECTOR E.C. T.C. F.I.D. X
Scavenger Rate ml./min.
Sens. 256 K1 Rec.Range 1 mv.
SAMPLE BP-benz, DPH, DCB Size 6 µl
Solvent MeOH Concn. 100 ppm
DPH 1
DPH 2
Benzidine
DCB

Inj. Figure 3. Gas chromatogram of benzidines using FID on OV-225 temperature programmed from 140° to 240° at 8°/min.

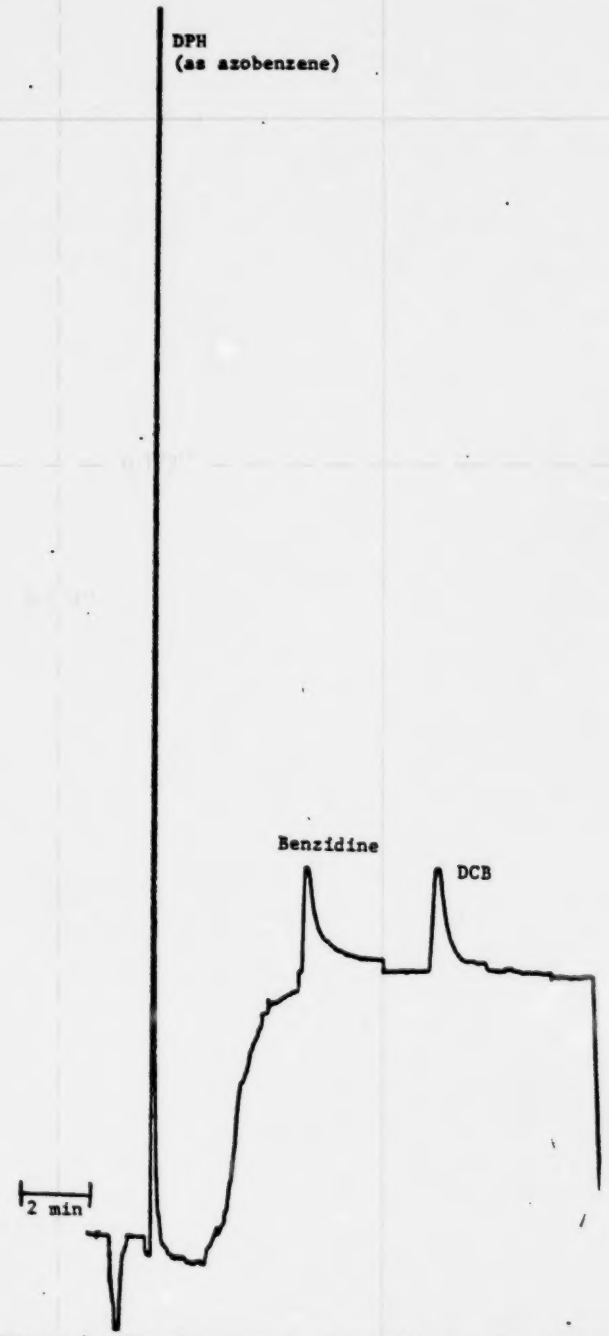


Figure 4. Gas chromatogram of benzidines using AFD on SP2250DB temperature programmed from 140°C to 240°C at 8°/min.

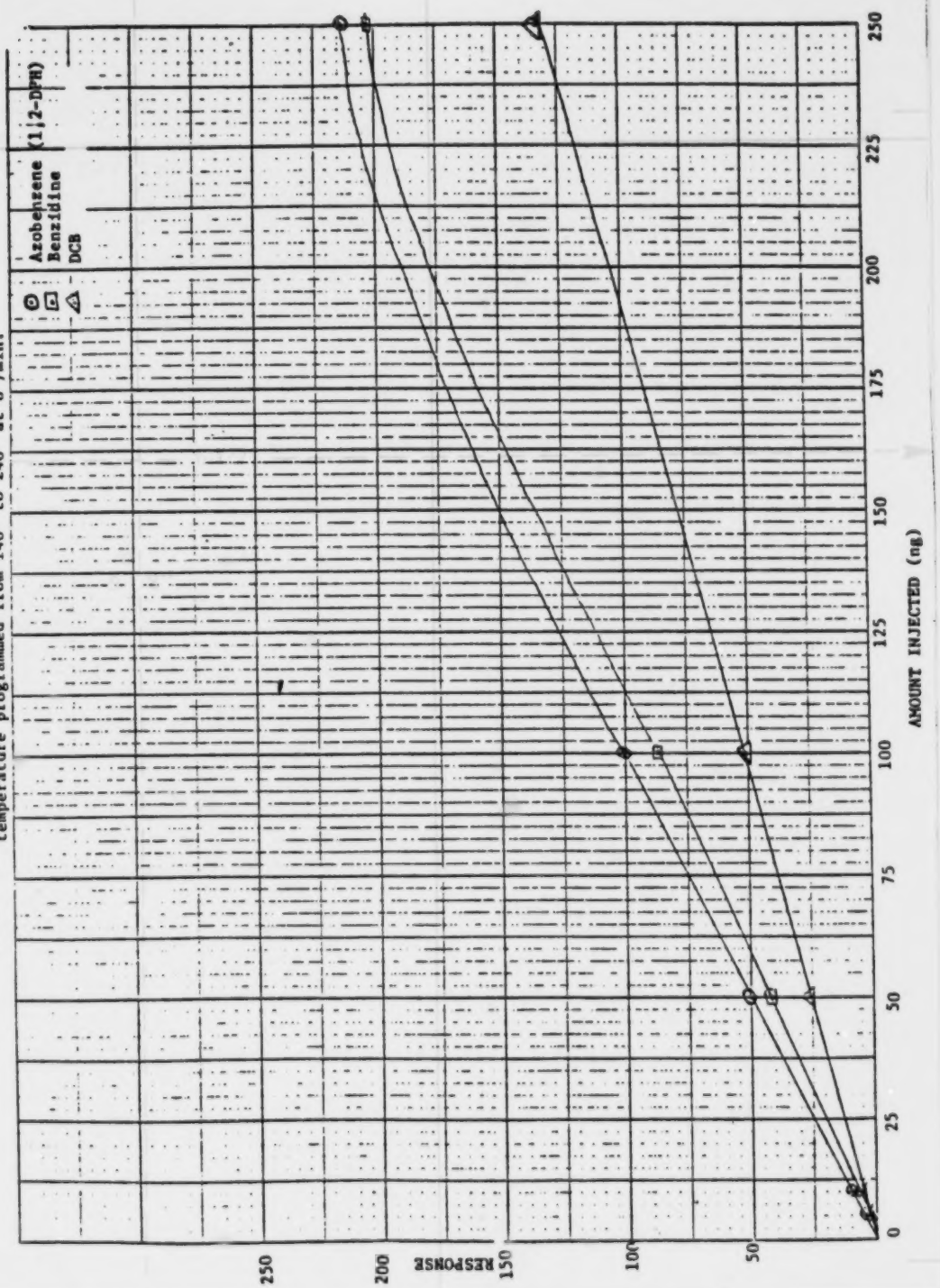


Figure 5. Linearity curve for benzidines using APD on SP2250DB temperature programmed from 140° to 240° at 8°/min.

Figure 6. GC-MS RCG of DPH.

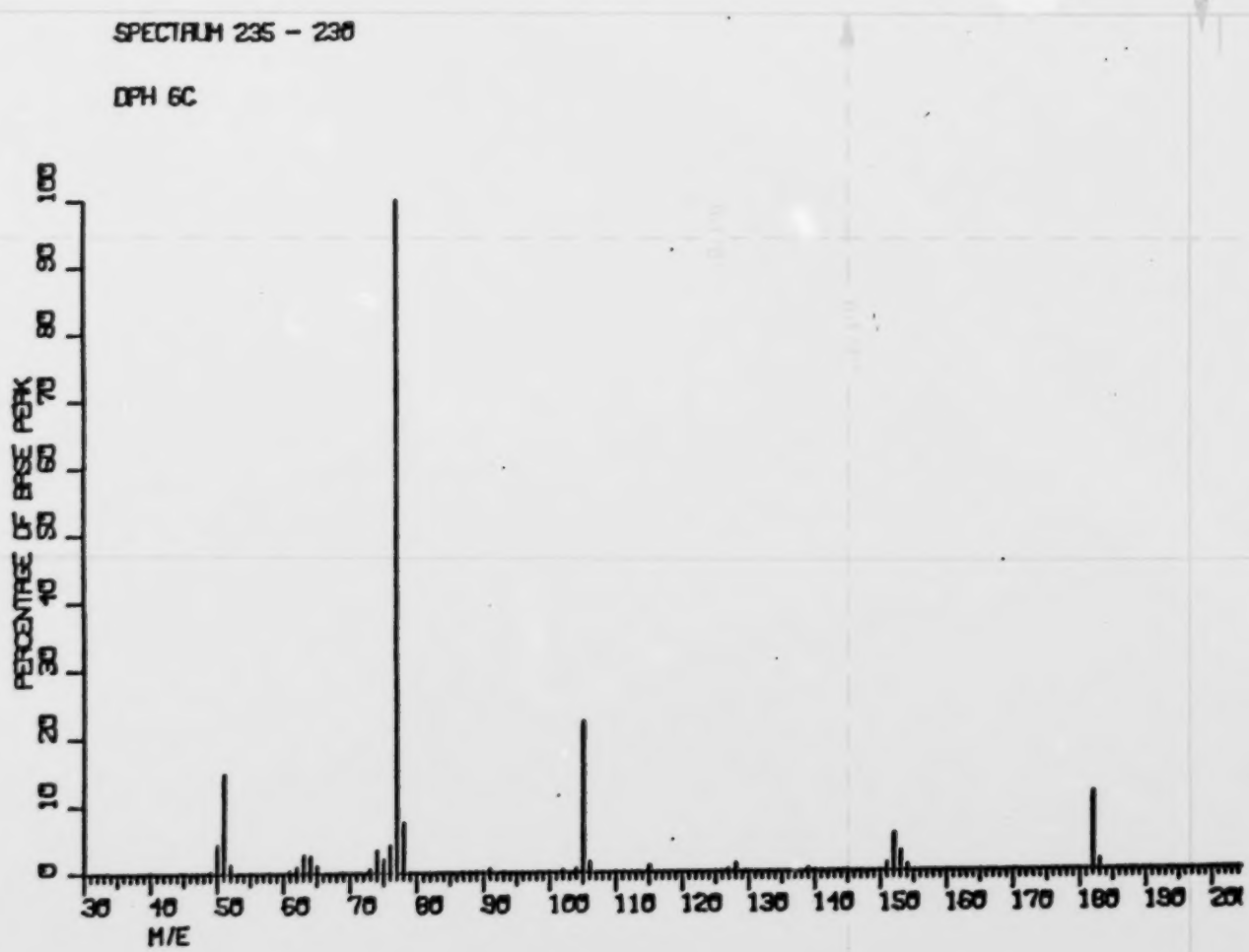


Figure 7. Mass spectrum of GC-MS peak from Figure 6.

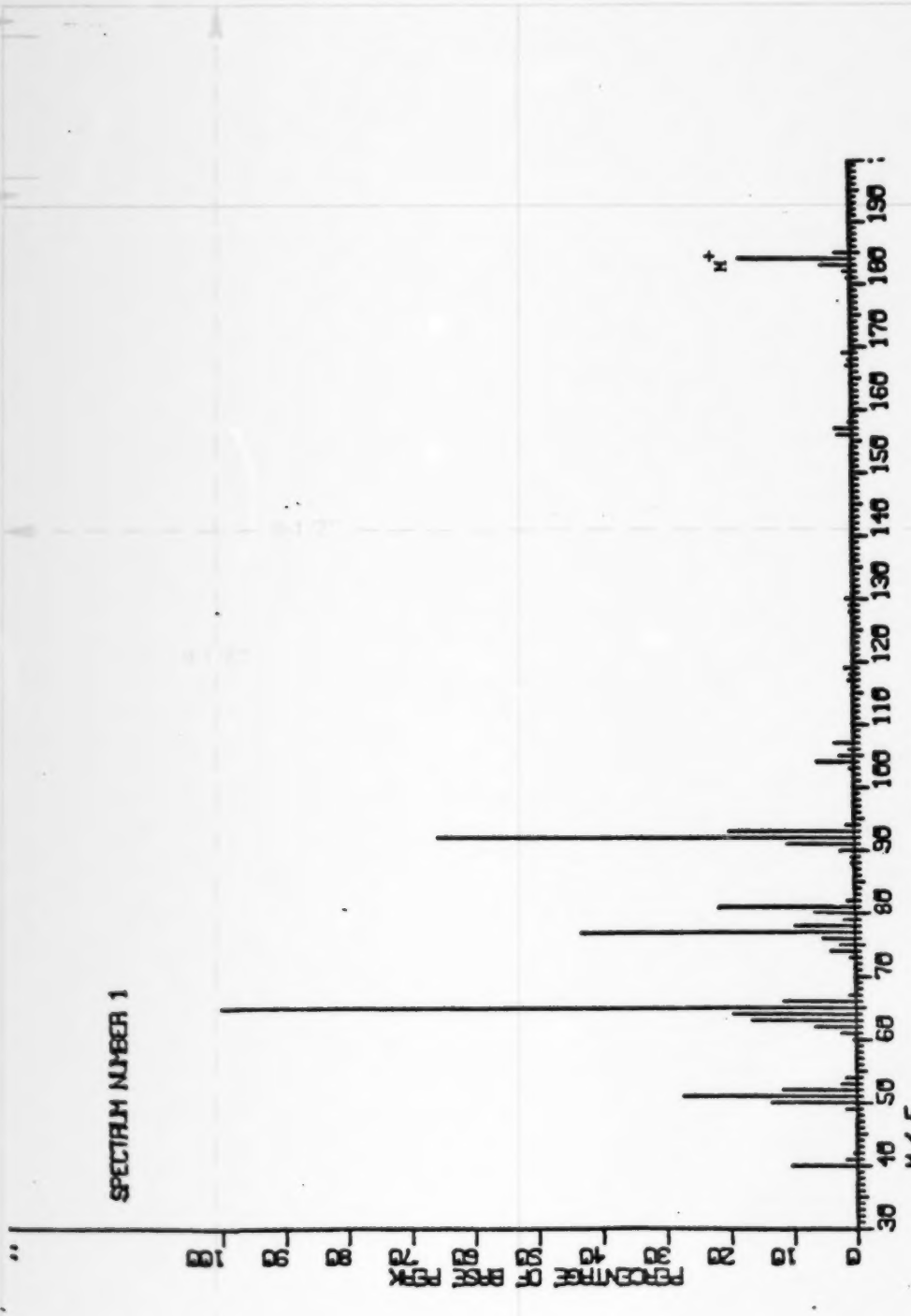


Figure 8. Mass spectrum of DPH via solid probe.

shown in Figure 9. It is apparent, therefore, that DPH instantaneously decomposes to azobenzene in the GC injection port.

It is interesting to note that only one peak resulted from DPH injection. Occasionally, it was found that a second, later eluting peak was present (Figures 1-3), but this was not always the case. This additional component may have been a solution decomposition product.

GC/ECD

GC/ECD was investigated for detection of benzidines after derivatization with a fluoroacyl group. All analyses were performed using the GC-FID conditions listed in the Experimental Section and many potential derivatization procedures investigated are discussed there, as well. Numerous problems were encountered using this approach, the most serious of which was the production of multiple peaks and the incomplete reaction of DPH with all of the reagents studied. Benzidine reacted well with the HFBI but not any of the anhydrides or the TFI. DCB derivatized the best of the three amines and failed to form a derivative only with TFA. Figure 10 illustrates the derivatization problems using DPH. Similar results were obtained using both the anhydrides and imidazoles. Figure 11 shows the formation of the DCB-HFBA derivative with good yield whereas Figure 12 shows the relatively poor yield (about 10%) of benzidine-HFBA derivative using the previously described conditions. No underivatized amine was present in either case, so some decomposition must have occurred in the benzidine derivatization reaction. Figures 13 and 14 show the successful derivatization (with good yield) of both benzidine and DCB using HFBI. The relatively high background using HFBI is due to the direct injection of the reaction mixture and may perhaps be reduced by partitioning between hexane/water prior to injection of the hexane layer. As stated earlier, HFBI derivatization of DPH was not successful since no derivative formation was observed.

On the basis of these results, this approach did not seem useful for the analysis of benzidines and was not further investigated, since an alternate (HPLC/EC) technique was working quite well. However, it appears that HFBI would be the best derivatizing reagent to use for benzidine and DCB, if one wished to assay these compounds by GC/ECD.

HPLC With Electrochemical Detection

This approach proved useful almost immediately, so, following a short period of column evaluation, a set of parameters was selected which gave excellent resolution, sensitivity, and reproducibility. Several reversed phase and ion exchange columns were preliminarily evaluated, including: Zorbax ODS and CN, μ Bondapak C-18, Lichrosorb RP-18, RP-8, and RP-2, Sperisorb ODS, and Whatman Partisil SCX. The Lichrosorb columns were prepared at Battelle whereas the other columns were purchased commercially. All the ODS, CN, and RP-8 packings gave poor efficiency for the amines -- less than 2000 plates in all cases -- although they gave efficiencies of greater than 10,000 plates for anthracene, which is commonly used for determining column efficiency. The Whatman SCX column was very poor in that

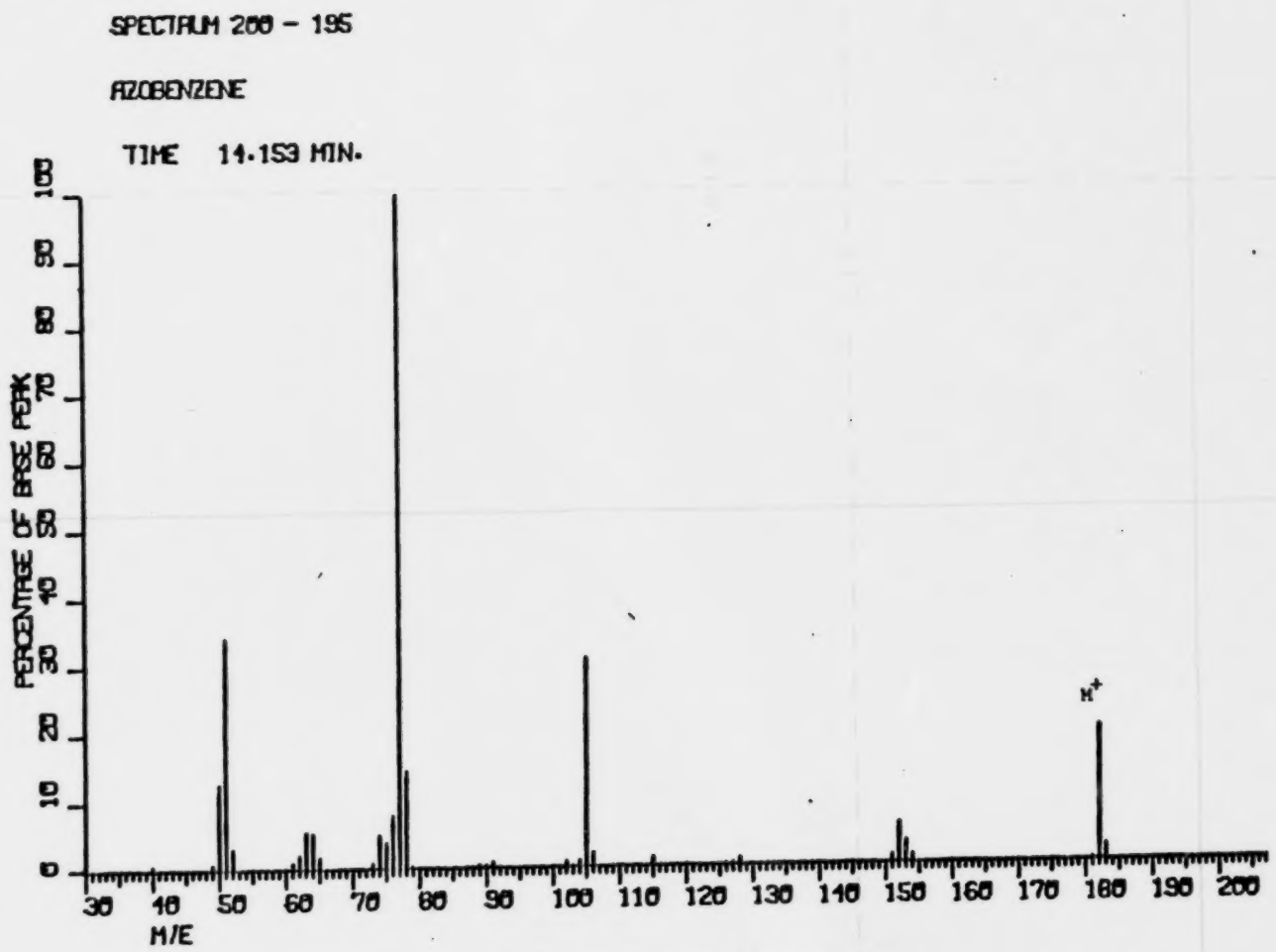


Figure 9. Azobenzene mass spectrum from GC-MS system.

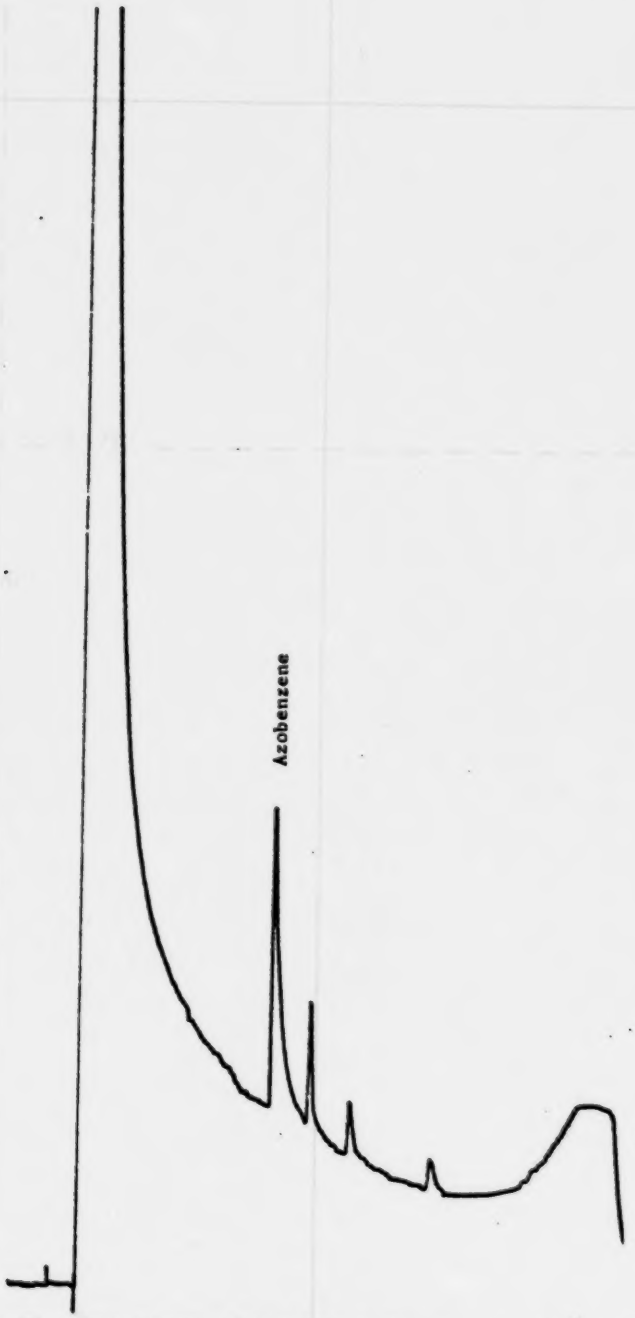


Figure 10. Chromatogram of DPH-HFBA derivatization product using GC/FID programmed from 100°-300° at 8°/minute.

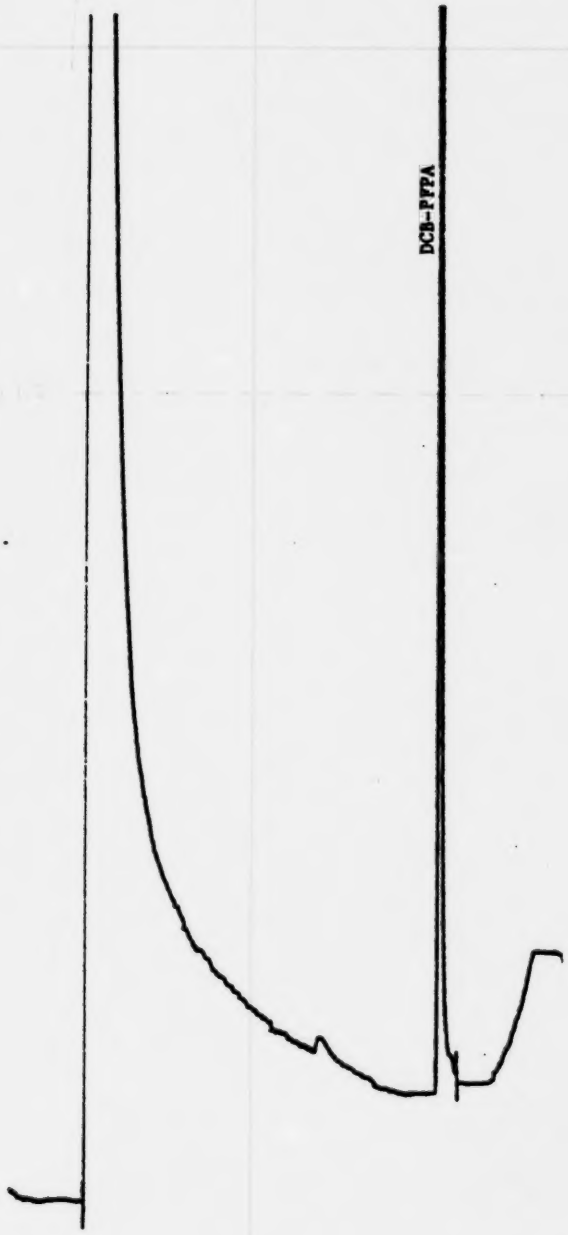


Figure 11. Chromatogram of DCB-PFPA derivatization product using GC/FID programmed from 100°-300° at 8°/minute.

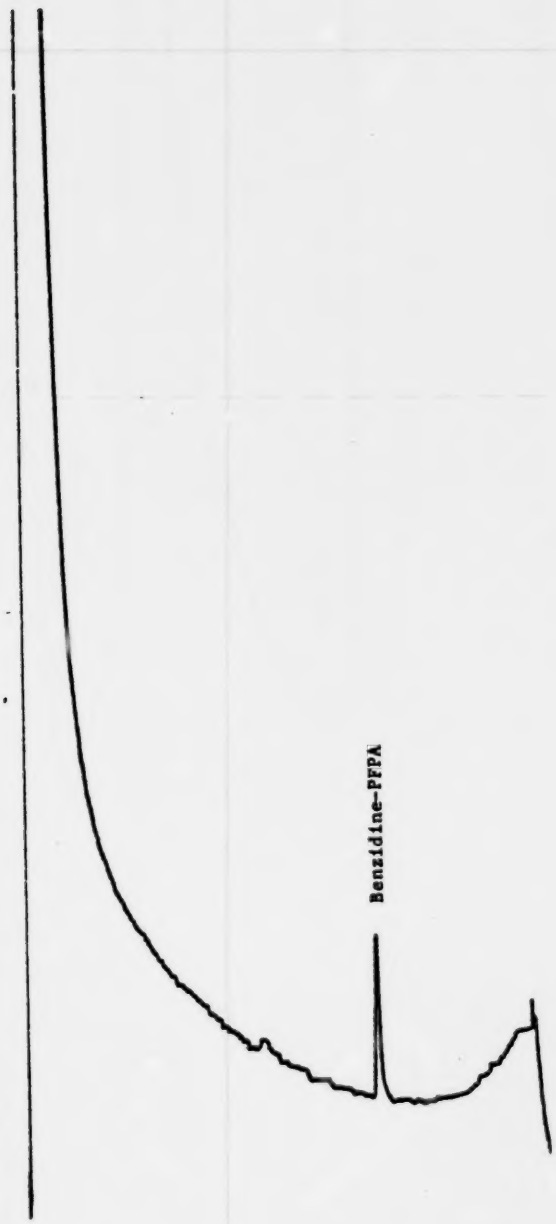


Figure 12. Chromatogram of benzidine-PFPA derivatization product using GC/FID programmed from 100°-300° at 8°/minute.

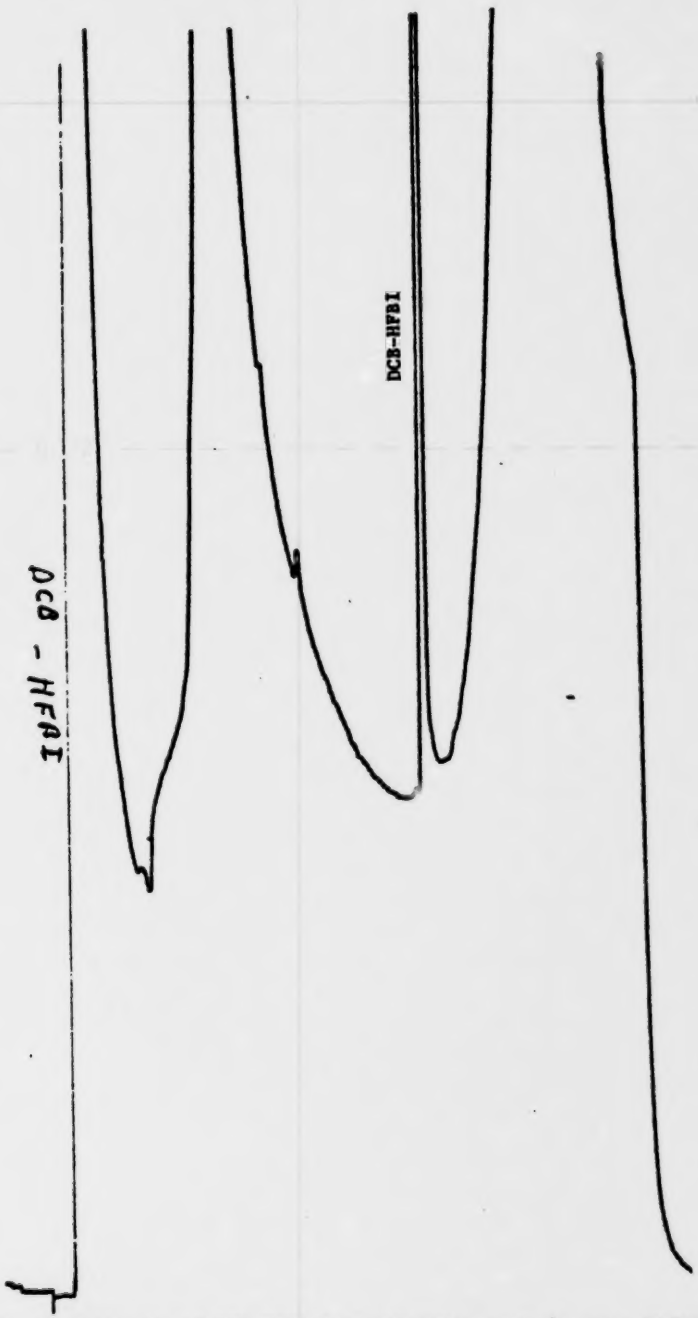


Figure 13. Chromatogram of DCB-HFBI derivatization product using GC/FID programmed from 100°-300° at 8°/minute.

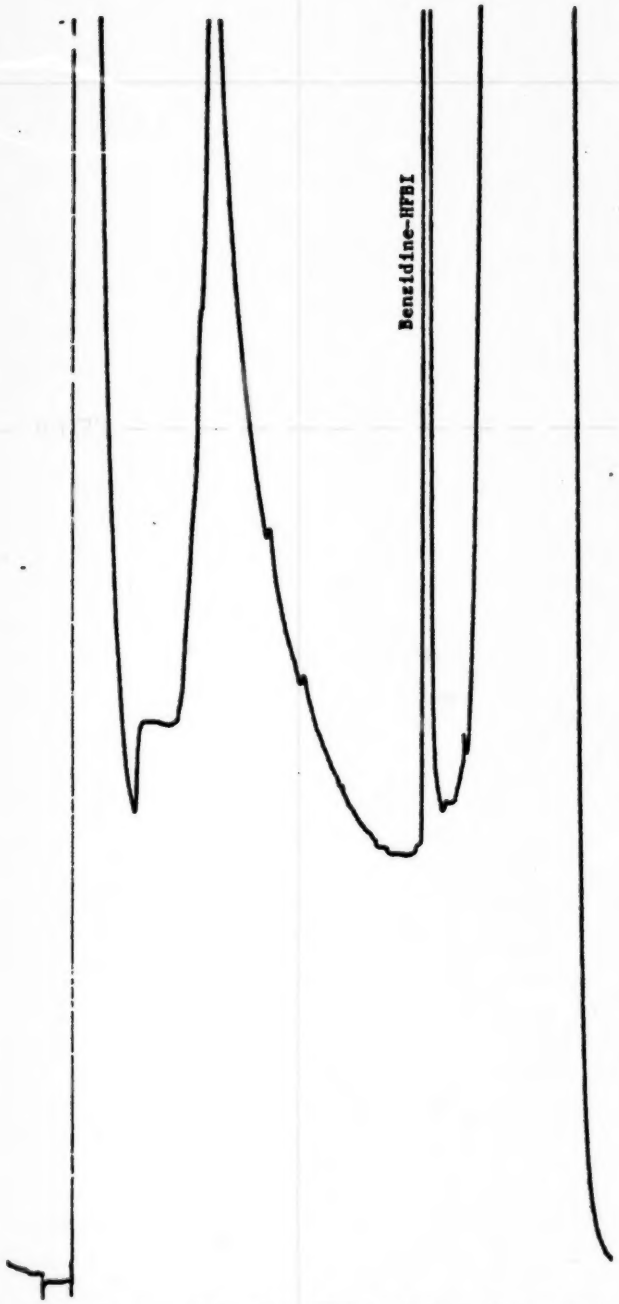


Figure 14. Chromatogram of benzidine-HFBI derivatization product using GC/FID programmed from 100°-300° at 8°/minute.

it gave badly tailing peaks. The RP-2 column was found to give excellent efficiency for the amines -- greater than 6000 plates. Better resolution of the DCB and DPH was achieved using acetonitrile instead of methanol under the conditions evaluated. Table 2 summarizes the retention properties of the three compounds under several of the conditions investigated.

Based on the results mentioned above, a set of HPLC conditions was selected which gave optimum resolution and sensitivity for the compounds of interest. These conditions are listed below:

Column -- RP-2 5 micron 25 cm x 4.6 mm I.D.
Mobile phase -- 50% acetonitrile - 50%
sodium acetate buffer 0.1M
pH 4.7
Detector -- Electrochemical (glassy carbon)
3 mm diameter, Bioanalytical Systems
model LC2
Detector potential -- 0.8V
Injection volume -- 25 microliters

The chromatogram in Figure 15 shows the separation using the above conditions. Using the electrochemical detector, the minimum detectable quantities were 0.05 ng for benzidine, 0.1 ng for dichlorobenzidine, and 0.3 ng for DPH at a signal to noise ratio of five to one. Linear response for the components is obtained from 0.1 - 400 nanograms injected for DCB and benzidine (a linear dynamic range of 10^3) as shown in Figure 16. Above 400 nanograms, a rapid fall from linearity is observed for DCB and benzidine. Precision of peak heights for eight replicate injections of benzidine was ±2.8 percent at the 50 nanogram level.

The electrode potential (0.8V) was chosen based on our study of response versus electrode potential for each of the compounds, the results of which are shown in Figure 17.

This study shows that full (diffusion controlled) response is achieved for each compound at 0.8V versus Ag/Ag Cl and above.

For confirmation purposes, an alternate column and mobile phase composition was found to be suitable as listed below:

Column - Sperisorb ODS, 5 micron particle diameter,
4.6 mm I.D. x 25 cm long
Mobile phase - 70% acetonitrile, 30% pH 4.7, .1M
sodium acetate buffer.

Under these conditions, the elution time for benzidine was 7.1 minutes and for DCB was 22 minutes. The retention time for DPH was not determined using these conditions.

SOLVENT STABILITY STUDIES

The stability of the three amines in water miscible solvents was studied over a 9-day period. For DCB and benzidine acetonitrile and methanol were

TABLE 2. RETENTION TIMES FOR BENZIDINES VARIOUS HPLC CONDITIONS

Stationary phase	Mobile phase	Retention time (minutes)*		
		Benzidine	DCB	DPH
RP-2, 4.6 mm x 25 cm	50% Acetonitrile, 50% .1M Acetate Buffer (pH 4.7)	6.1	11.8	12.2
RP-2, 4.6 mm x 25 cm	50% Methanol, 50% .1M Acetate Buffer (pH 4.7)	6.6	18.0	18.0
Spherisorb ODS, 4.6 mm x 25 cm	70% Acetonitrile 30% .1M Acetate Buffer	7.1	22.0	--

* At a flow rate of 0.8 mL/minute.

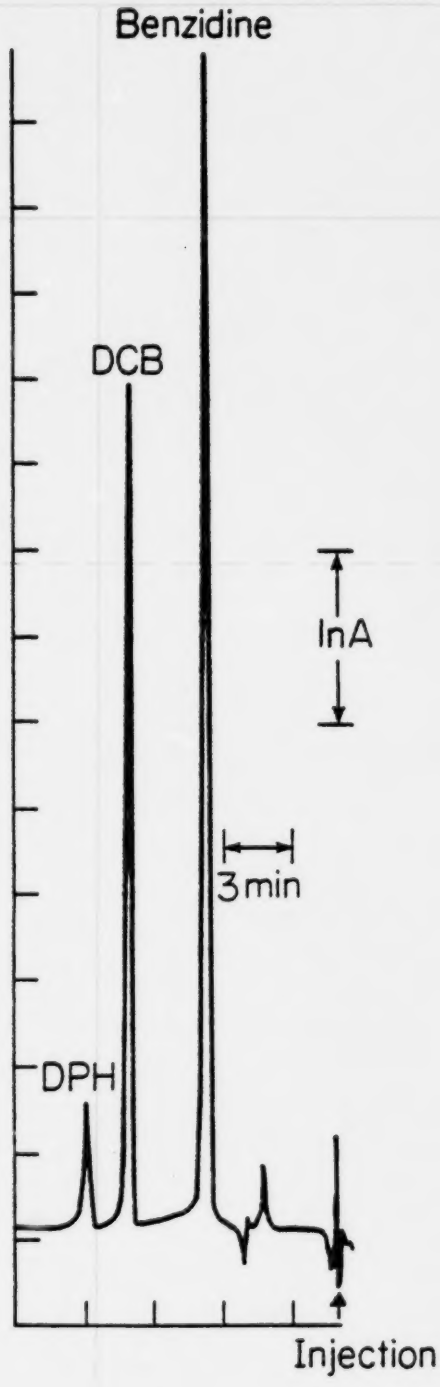
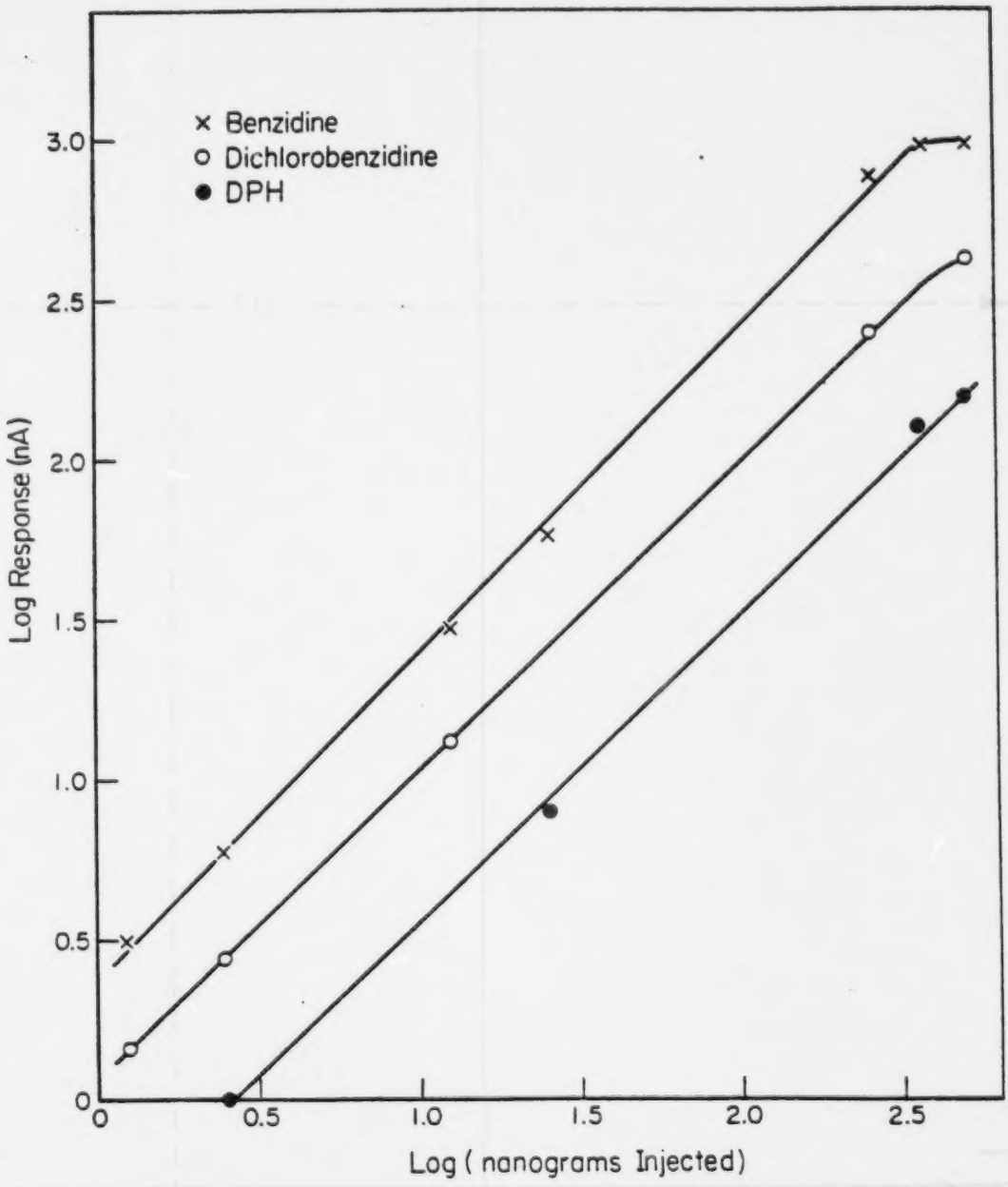


Figure 15. HPLC/EC chromatogram for three nanograms each of DCB and DPH.

Figure 16. Linearity curve for benzidines using HPLC with electrochemical detection.



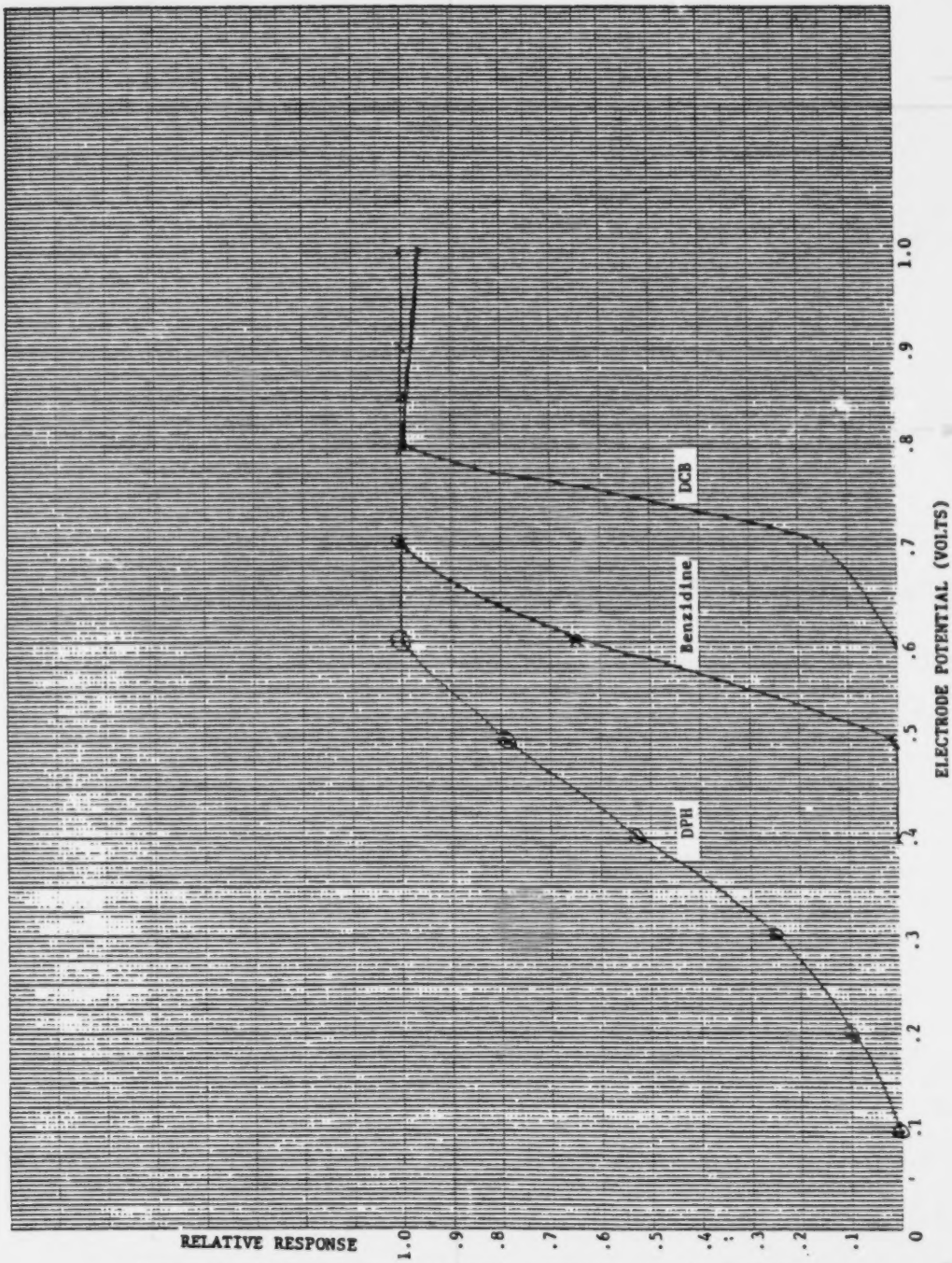


Figure 17. Response versus electrode potential for the benzidines.

used and both components were completely stable over the 90-day period. DPH was found to decompose completely in 3 days or less in all solvents investigated, including benzene, methylene chloride, methanol, triethylamine, acetonitrile, and acetic acid. It is apparent that DPH standards must be prepared fresh daily and thus no further studies were conducted. The results for this study are given in Table 3.

The ANOVA analysis shows that a significant variation is present for the methanol, but not for the acetonitrile solutions, although the results show this variation not to be consistent or directional. Therefore, it would appear that, while either solvent is useful, acetonitrile is slightly better, in that it gave the most consistent results.

EXTRACTION STUDIES

The extraction methodology was found to be a very delicate area since numerous unanticipated problems developed which led to low recoveries, especially for benzidine and DPH. The stability of DPH was found to be a major problem which was not completely solved.

The following are some of the problems encountered:

1. Benzidine is substantially adsorbed on Na_2SO_4 during the drying step. The use of K_2CO_3 corrected this problem. Elimination of the drying step was found to be useful when using HPLC analysis.

2. Benzidine is heat labile so that Kuderna-Danish concentration techniques gave low recoveries. The use of rotary evaporation eliminated this problem. The recoveries using a variety of concentration techniques are shown in Table 4.

3. Decomposition of benzidine occurred when concentrating methylene chloride or chloroform. The addition of 15 percent MeOH prior to concentration stabilizes the benzidine.

Chloroform with ethanol preservative and methylene chloride were evaluated as solvents for the extraction studies, the results of which are shown in Table 5 along with the ANOVA results. Generally, chloroform at pH 7 was found to give a more efficient extraction, especially for benzidine. As expected, no benzidine was recovered at pH 2, whereas the less basic DCB was extracted at all pH values (pH 2 data was not included in the ANOVA analysis since the effect was obvious). DPH degraded readily in both aqueous and organic media and although both chloroform and methylene chloride gave about 70% recoveries at pH 10, no set of extraction parameters was found which gave greater than about 70% recovery. At pH 2, DPH was found to degrade to benzidine to some extent. Based on this data, it was concluded that chloroform is a satisfactory solvent for the extraction of benzidines.

STORAGE STABILITY STUDIES

The stability of the benzidines in water under a variety of storage conditions was investigated. Two temperatures, 4 C and room temperature,

TABLE 3. SOLVENT STABILITY

Methanol	% Stability Benzidine	DCB	DPH
0	85	90	81
	87	90	81
	84	90	81
	85 ± 2*	90	81
30	108	105	0
	94	93	
	95	105	
	99 ± 8	101 ± 7	
60	92	87	0
	95	87	
	93	86	
	93 ± 2	87 ± 1	
90	93	92	0
	97	90	
	95	90	
	95 ± 3	91 ± 1	
Acetonitrile			
0	100	102	109
	98	95	100
	101	98	80
	100 ± 2	98 ± 4	96 ± 15
30	96	88	0
	102	92	
	93	99	
	97 ± 5	93 ± 5	
60	100	98	0
	100	98	
	103	98	
	101 ± 2	98	
90	98	101	0
	99	100	
	98	101	
	98 ± 1	101 ± 1	

ANOVA Analysis

	Benzidine	DCB
Methanol	5.671**	9.277**
Acetonitrile	1.418	1.854
F.95	4.07	4.07

* Average ± Standard Deviation.
**Significant difference at the 95% confidence level.

TABLE 4. RECOVERIES USING VARIOUS MEANS OF CONCENTRATION

Mode	% Recovery Benzidine	Dichlorobenzidine
Vortex evaporation	96 ± 6*	93 ± 12
Nitrogen evaporation	91 ± 6	95 ± 17
K-D (tube heater)	64 ± 8	79 ± 12
K-D (water Bath)	80 ± 13	93 ± 17
Full EMSL protocol	47 ± 28	78 ± 15

* Standard deviation for triplicate analyses.

TABLE 5. EXTRACTION STUDIES

Methylene Chloride	% Recovery Benzidine	DCB	DPH
pH2	0	80	9
	0	69	33
	0	77	0
	0	75 ± 7 *	14 ± 16
pH 7	68	99	74
	81	84	82
	81	99	58
	76	104	60
	76 ± 8	96 ± 9	68 ± 17
pH 10	92	101	75
	103	104	79
	80	82	82
	92 ± 13	96 ± 12	79 ± 4
Chloroform			
pH 2	0	93	35
	0	84	28
	0	89	41
	0	92 ± 3	35 ± 19
pH 7	87	92	69
	89	92	70
	88	95	64
	92	99	37
	89 ± 2	94 ± 4	60 ± 24
pH 10	79	88	81
	90	98	65
	88	94	67
	86 ± 7	93 ± 5	67 ± 16

ANOVA Analysis (Excluding pH 2)

	Benzidine	DCB
Effect of pH (7 vs 10)		
Chloroform	1.15	0.14
Methylene Chloride	5.22	0.01
F .95	6.61	6.61
Effect of Solvent		
pH 7	14.7**	0.19
pH 10	0.22	0.04
F .95	5.99	5.99

* Average ± standard deviation.
** Significant difference at the 95% confidence level.

two chlorine levels, 0 and 2 ppm as NaOCl, and three pH levels of pH 2, 7, and 10 were evaluated. The results of this study are shown in Table 6.

None of the amines were detectable in the solutions to which chlorine was added. A pH of 2 was found to give the best results for benzidine and DCB. However, at pH 2 DPH degrades to benzidine, thus creating an undesired artifact. This problem can be overcome by employing pH 4.7 acetate buffer where both benzidine and DCB are well preserved, and DPH degrades to two unidentified components, not to benzidine. Figures 18 and 19 illustrate the degradation of DPH at each of the pHs studied. At pH 4.7, two peaks were obtained which did not interfere with DCB or benzidine and neither of which was DPH (based on chromatographic retention times).

Based on these results, it was concluded that a pH between 2 and 4 is best for preservation of the benzidines. Some artifacts due to DPH decomposition to benzidine are likely at pH 2 and below if DPH is present in the sample. Addition of a reducing agent (such as Na_2SO_3) to destroy chlorine is required.

DPH was found to be very unstable in wastewater, specifically secondary sewage. As shown in Figure 20, DPH, spiked at the 100 ppb level, disappears with a half life of about 15 minutes in the presence of oxygen and about 60 minutes with oxygen removed. This result indicates that DPH analysis in wastewater is virtually meaningless, since the DPH level determined cannot be directly related to the DPH in the sample at the time of collection.

WASTEWATER ANALYSIS

As stated in the Experimental Section, eight water samples were selected for analysis, some of which were assayed using the method described in that section and some of which were assayed by the technique described in the Appendix, which was adopted at the request of the EPA Project Officer. A few samples were assayed by both techniques. A description of the results for each sample follows. The analytical results for all the samples are tabulated in Table 7.

Both the approaches were evaluated using distilled water and comparable results were obtained, as shown in Table 7. DCB recovery was somewhat lower using the second approach, probably due to the fact that the volume ratio of 1M H_2SO_4 to chloroform is lower in the second method. (DCB is much less basic than benzidine and requires strong acid to extract from organic solution.)

Both the approaches were also evaluated for surface water (Sample 1, Table 7) spiked at the 10 ppb level for Method 1, and at the 4 ppb level for Method 2. Once again, comparable results were obtained using the two techniques. Recoveries were approximately the same as were obtained for distilled water, although storage for a seven day period at 4°C and pH 2 resulted in substantially lower recoveries. Based on the background level observed, MDLs are estimated to be 0.1 ppb for both benzidine and DCB for this sample (based on a 5:1 signal to noise ratio).

TABLE 6. STORAGE STABILITY

		% Recovery	
		Benzidine	DCB
pH 2	Room	80	90
		94	85
		87	88
	4°C	80	82
		80	82
		80	82
pH 7	Room	64	110
		55	89
		60	99
	4°C	79	97
		56	83
		68	90
pH 10	Room	70	80
		73	82
		72	81
	4°C	82	93
		38	44
		64	74
pH 4.7	4°C	72	81
		86	91
		79	86
ANOVA Analysis			
pH Effects			
Room		8.096	2.249
4°C		0.497	0.546
F .95		9.55	9.55
Temperature Effect			
pH 2		1.000	4.840
pH 7		0.420	0.567
pH 10		0.297	0.260
F .95		18.5	18.5

TABLE 7. DATA FOR WASTEWATER ANALYSES

Sample No.	Sample description	Method*		Background (ppb)	Spike (ppb)	Recovery 0 days	Recovery 7 days
	Distilled water	1	Benzidine	<.1	10	79 ± 4**	-----
			DCB	<.1	10	83 ± 6	-----
	Distilled water	2	Benzidine	<.1	4	70 ± 7	-----
			DCB	<.1	4	50 ± 5	-----
1	Surface water	1	Benzidine	<.1	10	70 ± 6	46 ± 3
			DCB	<.1	10	69 ± 6	39 ± 3
1	Surface water	2	Benzidine	<.1	4	63 ± 6	-----
			DCB	<.1	4	48 ± 5	-----
2	Secondary sewage	1	Benzidine	<.1	10	67 ± 4	-----
			DCB	<.1	10	70 ± 3	-----
2	Secondary sewage***	2	Benzidine	<.1	4	73 ± 8	60 ± 12
			DCB	<.1	4	63 ± 7	58 ± 6
3	Primary sewage	2	Benzidine	.1	1.0	64 ± 5	71 ± 21
			DCB	.1	1.0	55 ± 10	19 ± 10
4	Final effluent*** for organic chemicals plant	2	Benzidine	<.1	1.0	47 ± 46	52 ± 17
			DCB	<.1	1.0	89 ± 8	60 ± 21
5	Final effluent from plant producing benzidine based dyes	1	Benzidine	19 ± 6	100	70 ± 2	88 ± 3
			DCB	<1	100	49 ± 8	50 ± 6
6	Final effluent from plant producing benzidine based dyes	1	Benzidine	17 ± 4	100	48 ± 8	18 ± 4
			DCB	<1	100	50 ± 6	54 ± 4
7	Final effluent*** from plant producing benzidine based dyes	2	Benzidine	12 ± 3	50	79 ± 14	47 ± 8
			DCB	<1	50	78 ± 7	23 ± 7
8	Process effluent*** from oxidation process in plant producing benzidine based dyes	2	Benzidine	<1	10	82 ± 3	75 ± 7
			DCB	<1	10	98 ± 5	95 ± 3

* Method 1 - Described in Experimental Section. Method 2 - Described in the Appendix.
** Mean ± standard deviation.
*** Stored for 48 hours instead of seven days.

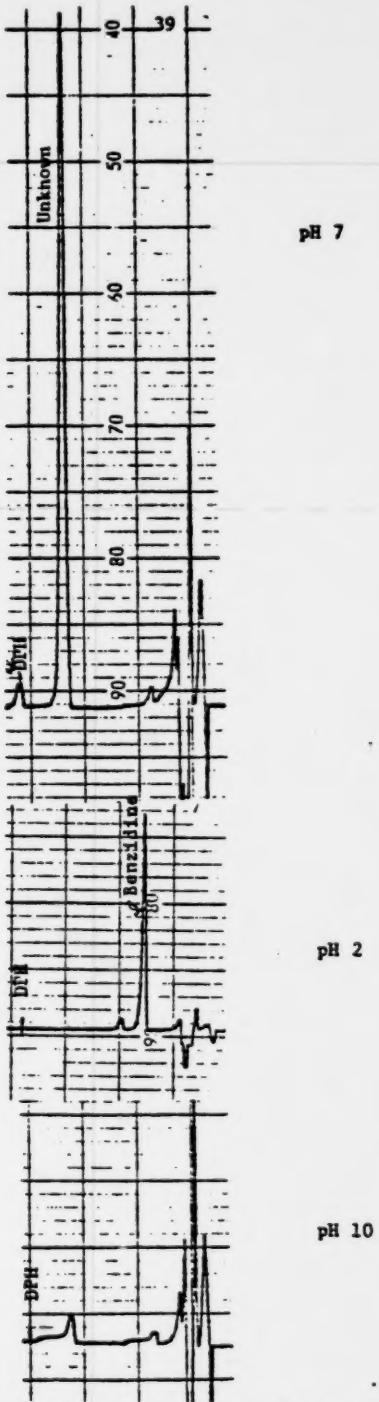


Figure 18. Results of preservation studies on DPH.

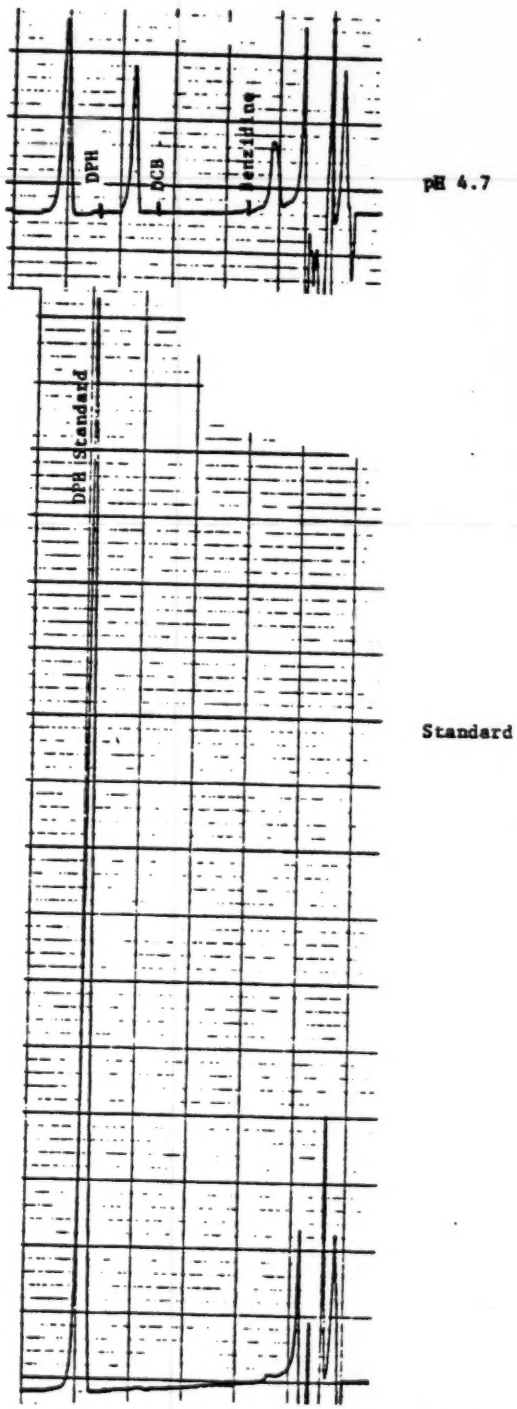


Figure 19. Results from preservation of DPH at pH 4.7.

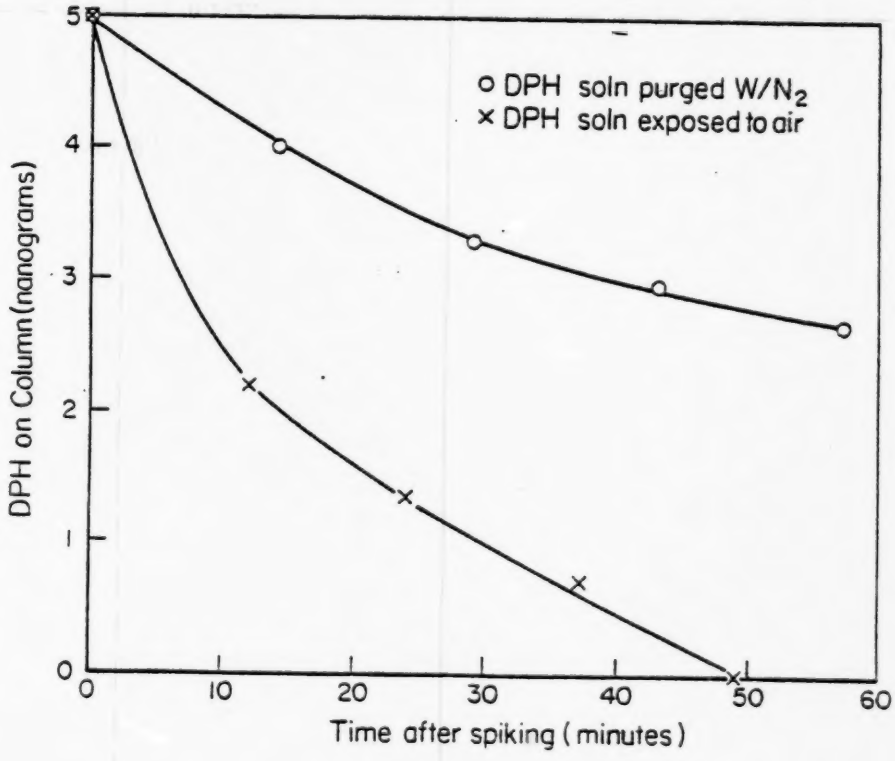


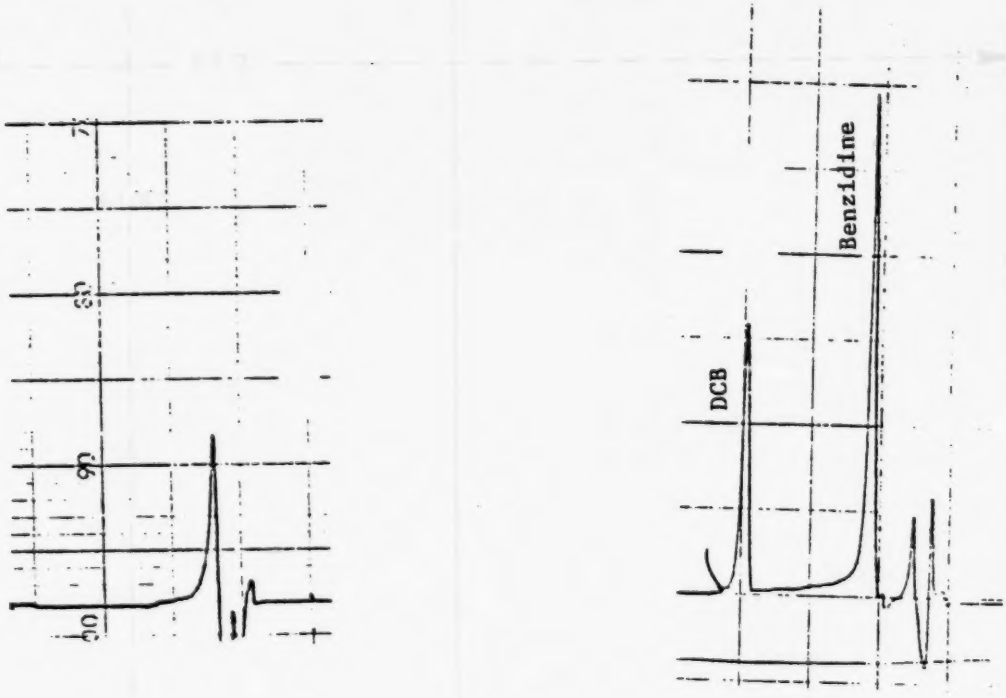
Figure 20. DPH degradation in a municipal sewage sample spiked with 100 ppb of DPH.

A secondary sewage effluent was also assayed by both methods, again at the 10 and 4 ppb levels. Results obtained were comparable to those for the surface water sample. Storge of this effluent, spiked at the 4 ppb, for 48 hours at 4°C and pH 2 resulted in essentially no loss in recovery. The shorter storage period was used, since it was felt that the stability of benzidine in dilute aqueous solution was so unpredictable that samples should be assayed as soon as possible. Forty-eight hours appeared to be the shortest time period which could reasonably be expected for the completion of sample collection and transport to the analytical laboratory. Figure 21 shows a typical chromatogram for this sample unspiked and spiked at the 4 ppb level. MDLs are estimated to be 0.1 ppb for both DCB and benzidine in this sample.

Sample 3 was a primary sewage sample and was assayed using Method 2. Typical chromatographs for the unspiked and spiked (1 ppb) sample are shown in Figure 22. This spike level was chosen in order to validate the procedure at levels approaching the detection limit, although as a general practice higher spiking levels were used so that accuracy could be more easily determined and poor recoveries would still result in a measurable peak. As shown in Table 7, recoveries were quite good and precision was about ±10-20%. However, after storage for seven days, low recovery and poor reproducibility were observed for DCB, but not for benzidine. This result appears to indicate that DCB may be irreversibly bound to particulate matter, as has been recently observed,[6] since this sample had a large amount of suspended matter. MDLs for this sample are estimated to be 0.1 ppb for benzidine and DCB.

Sample 4 was a final effluent sample from a plant producing various organic chemicals such as nitrophenols, o-dichlorobenzene, chloroanilines, and nitrobenzenes. Samples 1-3 were selected for this study because they represent matrices which receive inputs from a diversity of industrial processes, and thus benzidine determination might be applied to such samples in the future. Sample 4, on the other hand, was selected because it represents a matrix suspected of containing various aromatic amines (anilines) and phenols which would be likely interferences for the HPLC/EC method (since they are readily oxidized). Sample 4 was run using Method 2 and was spiked at the 1 ppb level. Typical chromatograms for the spiked and unspiked sample are shown in Figure 23. Recovery and particularly precision for benzidine was poor both before and after storage (for 48 hours). Recovery and reproducibility for DCB was better than for benzidine, both before and after storage. The reason for this lack of reproducibility for this sample is not clear, although it may be due to chemical oxidation, since benzidine is much more easily oxidized than DCB. Based on the background levels observed, the MDL is estimated to be 0.1 ppb for both compounds in this sample.

Samples 5, 6, and 7 were essentially identical samples obtained from a plant producing benzidine based dyes. These samples were about 1 year old upon receipt and had been stored at pH 2 and 4°C during that period of time. All three samples were diluted 10:1 with distilled water prior to analysis. Samples 5 and 6 were assayed using Method 1, whereas 7 was assayed using Method 2. Typical chromatograms for Samples 5 and 6 unspiked



(a) (b)

Figure 21. HPLC chromatograms for Sample 2 unspiked (a) and spiked with 4 ppb of benzidine and DCB (b).

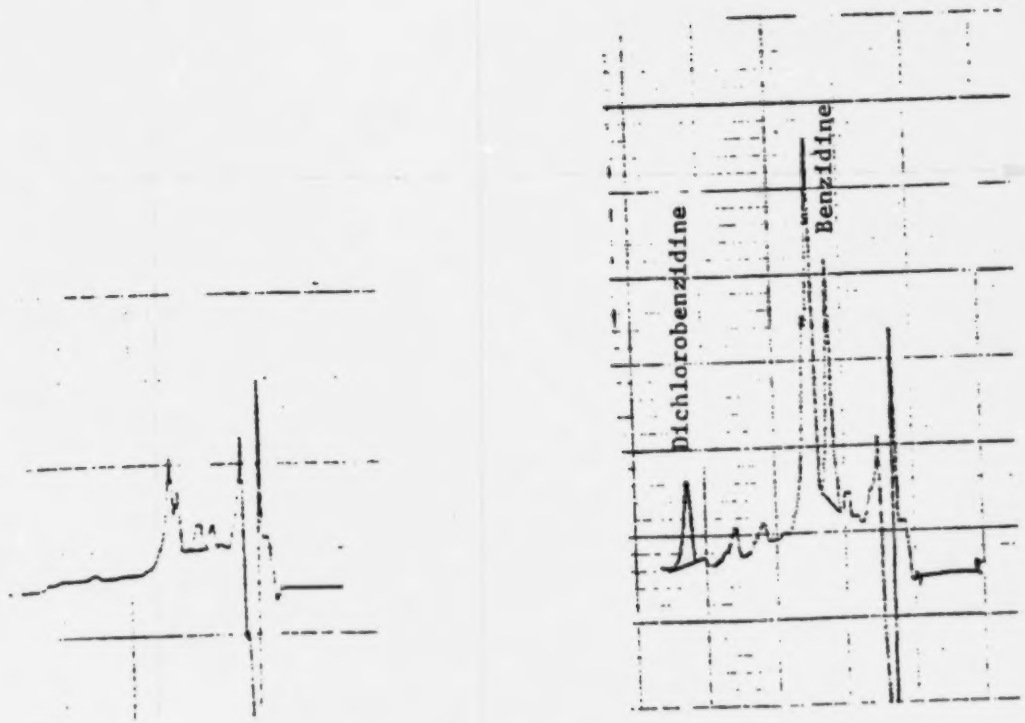
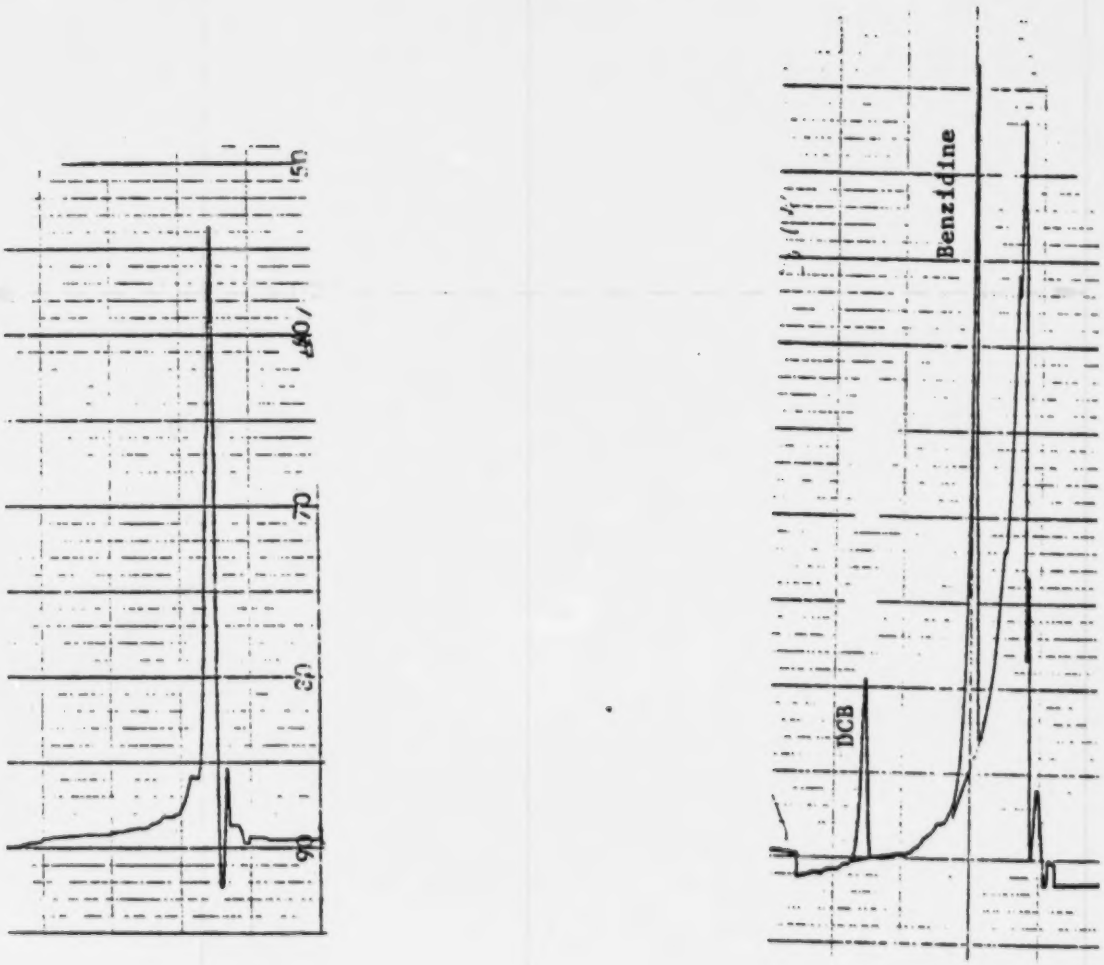


Figure 22. HPLC chromatogram for Sample 3 unspiked (a) and spiked with 1 ppb of benzidine and DCB (b).



(a) (b)

Figure 23. HPLC chromatogram for Sample 4 unspiked (a) and spiked with 1 ppb of benzidine and DCB (b).

and spiked with 100 ppb of benzidine and DCB are shown in Figures 24 and 25, respectively. Recoveries were on the order of 50-70% before storage and in most cases did not decrease after storage. However, for Sample 6 the apparent benzidine level dropped drastically and for Sample 7 DCB recovery decreased. The drop in benzidine level for Sample 6 was particularly disturbing since there was a detectable level of benzidine in the unspiked sample, which had been stored for over a year. It seemed possible therefore that the benzidine background was due to matrix interferences, rather than benzidine. Unfortunately, Samples 5 and 6 were exhausted so further experiments were conducted using Sample 7. Specifically, the extract from Sample 7 was injected at various electrode potentials to see if a lower electrode potential would result in a lower background for benzidine. Figure 26 shows the chromatograms for Sample 7 injected at 0.8 volts versus Ag/AgCl. Figure 27 shows chromatograms for the same sample at 0.6 volts versus Ag/AgCl. It is readily apparent that the reduction in potential greatly enhances the size of the benzidine peak relative to the other peaks. It is important to note that the apparent level of benzidine in the unspiked sample decreases in going from 0.8 to 0.6 volts. This indicates that an interfering component, with an oxidation potential greater than benzidine is present. However, it was still not certain whether the benzidine level at 0.6 volts was accurate. In order to check this, a second set of HPLC conditions was used as detailed below:

Column - Spherisorb - ODS, 5 micron particle diameter, 4.6 mm. I.D. x 25 cm long

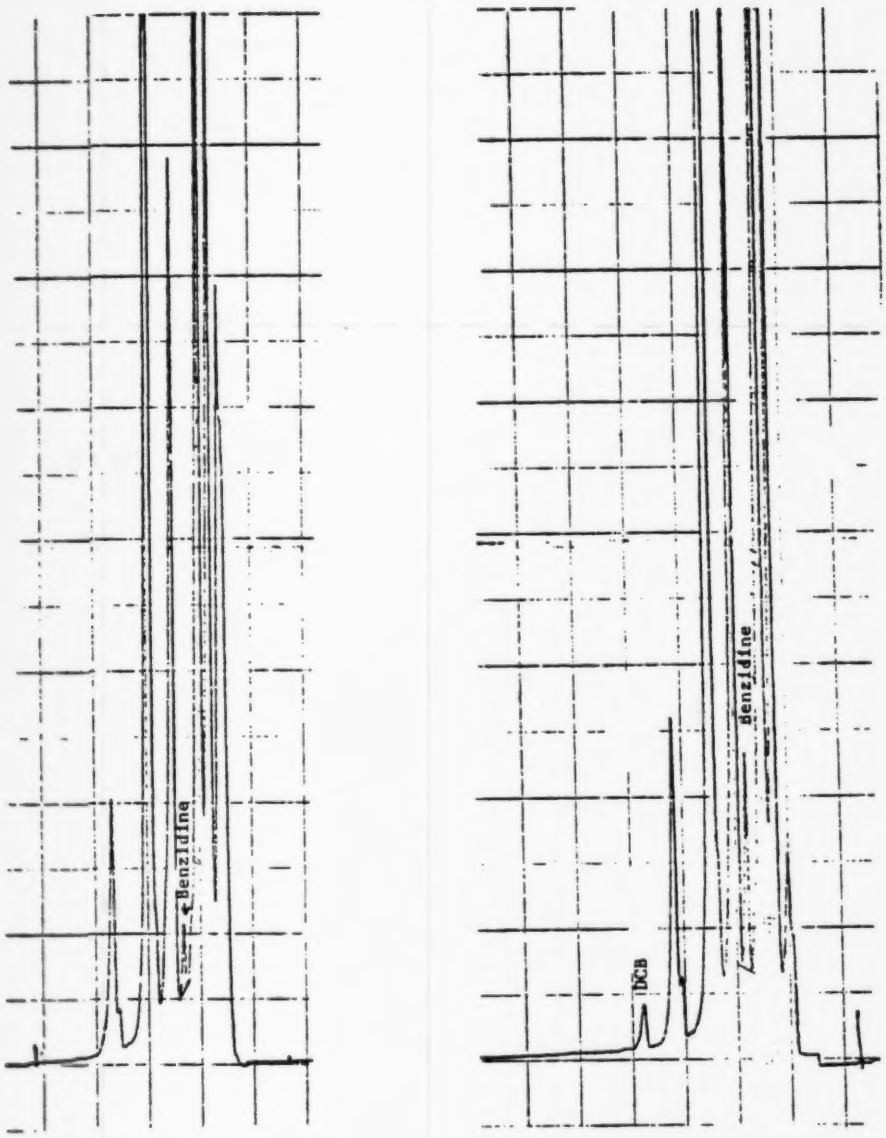
Mobile phase - 70% Acetonitrile, 30% .1M acetate buffer (pH 4.7)

Flow rate - 0.8 mL/minute

The chromatograms at 0.8 and 0.6 volts using these chromatographic conditions are shown in Figures 28 and 29, respectively. It is important to note that in the unspiked chromatogram in Figure 28 (0.8 volts) benzidine appears as a shoulder on a larger peak, whereas at 0.6 volts (Figure 29) benzidine is the larger of the two peaks, thus indicating that the earlier eluting peak is more difficult to oxidize than benzidine. Most importantly, the value obtained for benzidine using the original chromatographic conditions (12 ppb) correlates closely with the value obtained using the Sperisorb column (9 ppb) thus indicating that the peak probably is benzidine.

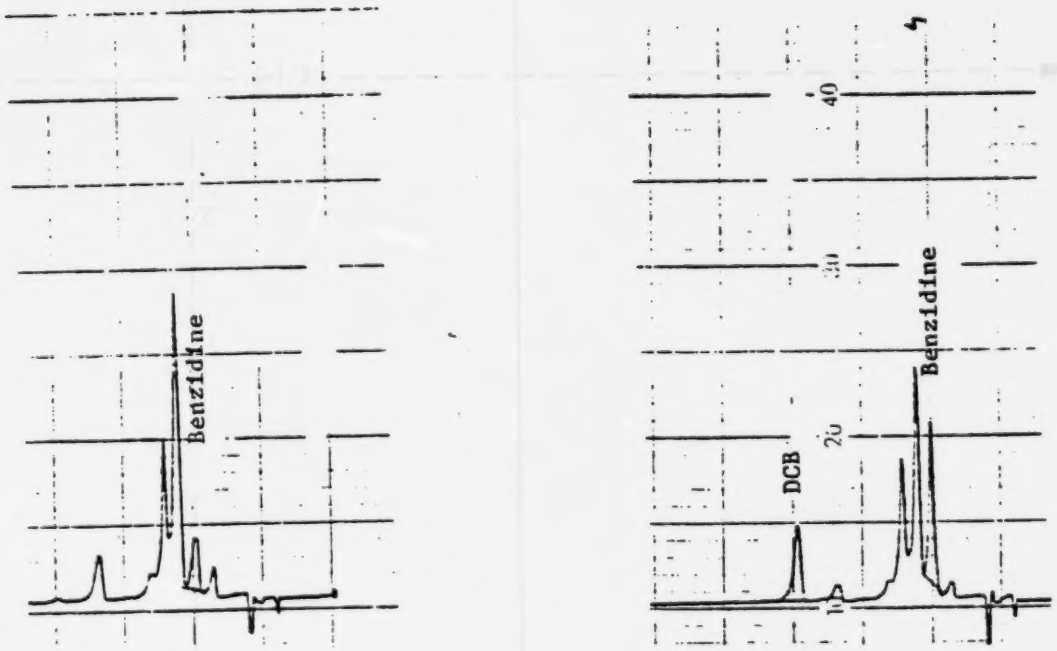
MDLs for DCB in samples 5, 6, and 7 are estimated to be 1 ppb. MDLs for benzidine are difficult to estimate but are considered to be approximately 1 ppb, based on the intensity of the neighboring peaks at the 0.6 volts potential.

The final sample, sample 8, run through the analytical scheme was an oxidation process effluent from the benzidine based dye plant from which samples 5, 6, and 7 were taken. This sample was also diluted 10 to 1 with distilled water before analysis. Due to a high background at 0.8 volts, benzidine was determined at 0.6 volts. Typical chromatograms for the unspiked and spiked sample at 0.8 and 0.6 volts are shown in Figures 30 and



(a) (b)

Figure 24. HPLC chromatograms for Sample 5 unspiked (a) and spiked with 100 ppb for benzidine and DCB (b).



(a) (b)

Figure 25. HPLC chromatogram for Sample 6 unspiked (a) and spiked with 100 ppb of benzidine and DCB (b).

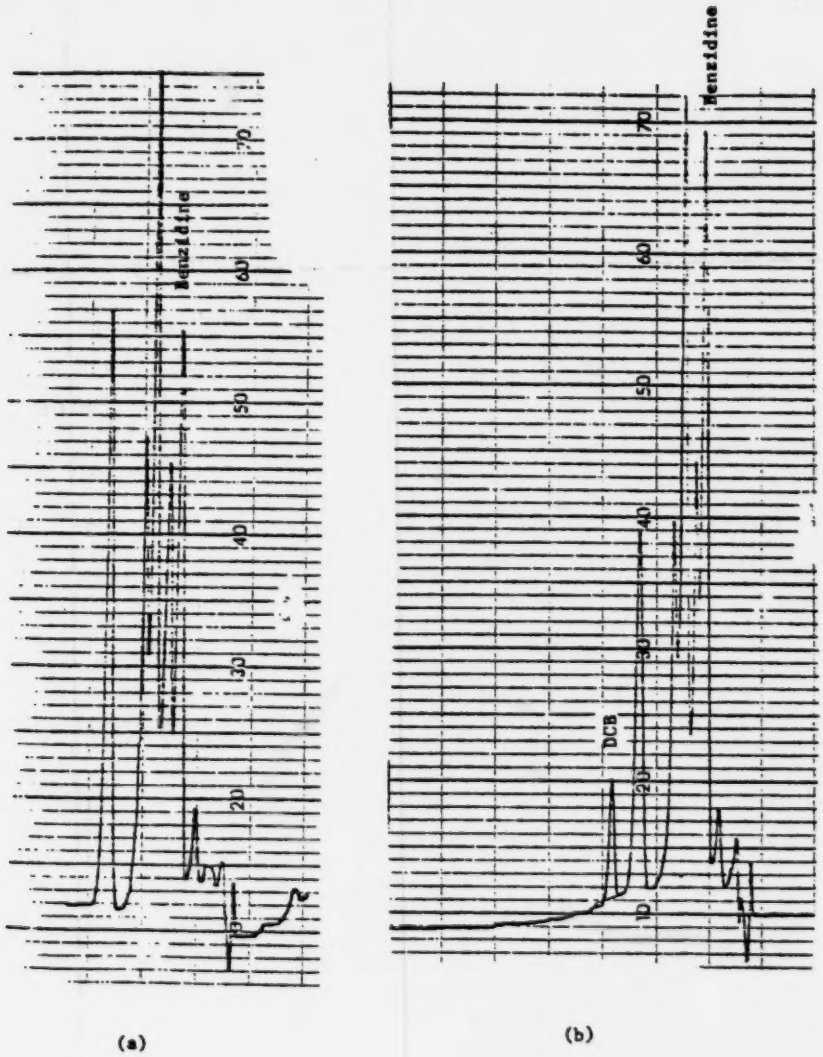
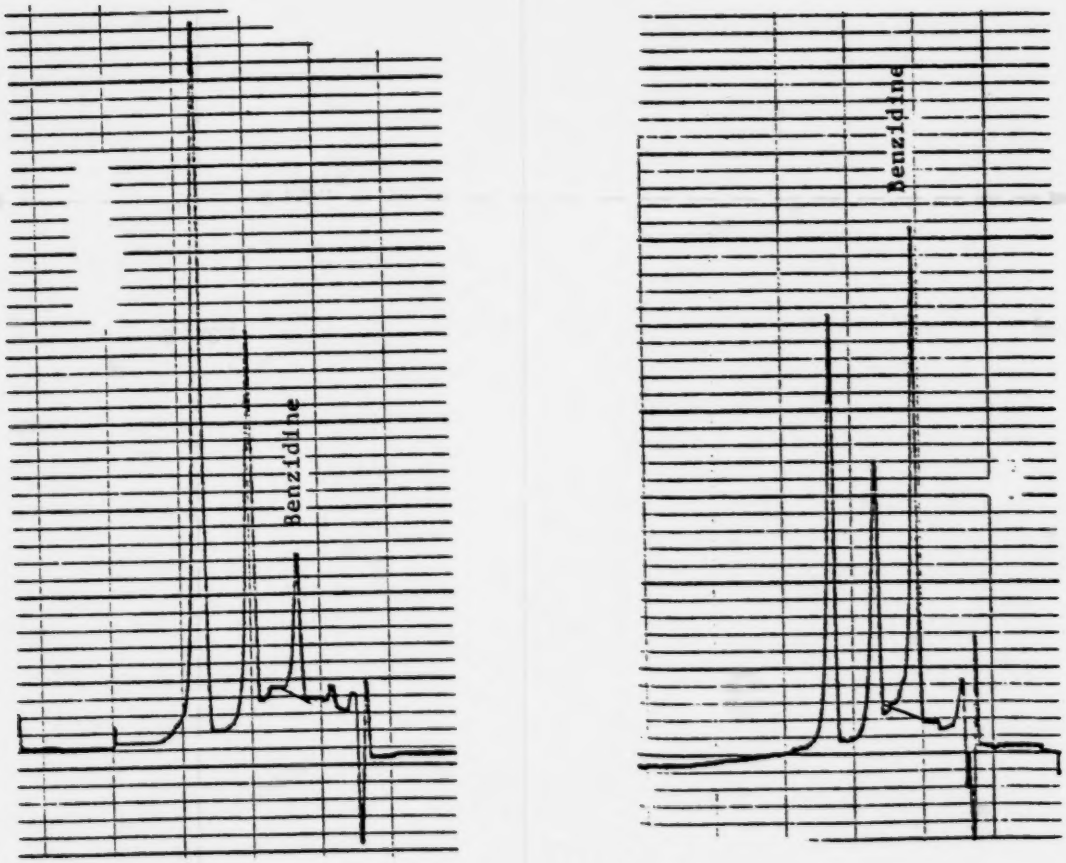


Figure 26. HPLC chromatograms for Sample 7 unspiked (a) and spiked with 50 ppb of benzidine and DCB (b) at 0.8 volts vs. Ag/AgCl.



(a) (b)

Figure 27. HPLC chromatograms for Sample 7 unspiked (a) and spiked with 50 ppb of benzidine and DCB (b) at 0.6 volts vs. Ag/AgCl.

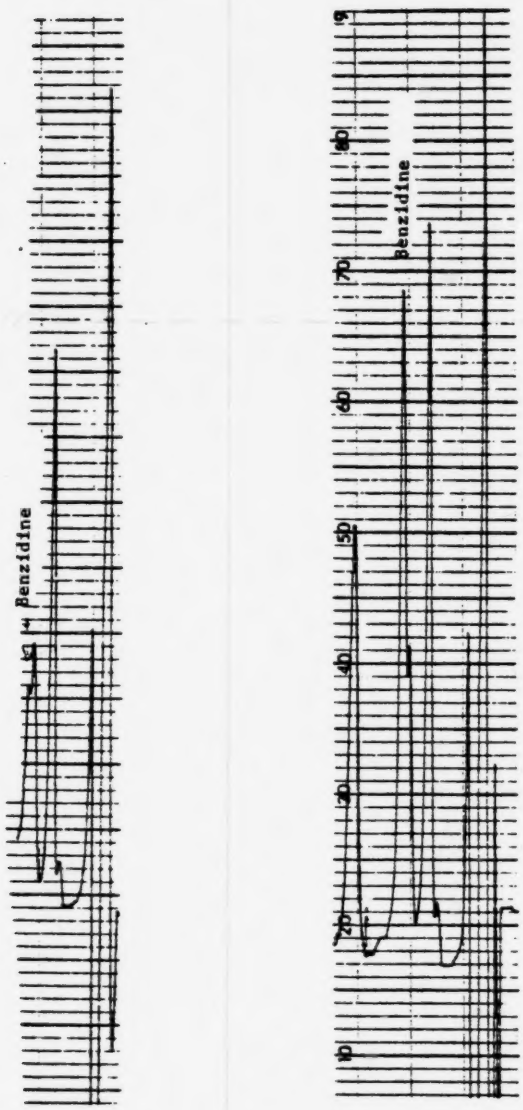


Figure 28. HPLC chromatogram of Sample 7 unspiked (a) and spiked with 50 ppb of benzidine and DCB (b) at 0.8 volts on a Spherisorb-ODS column.

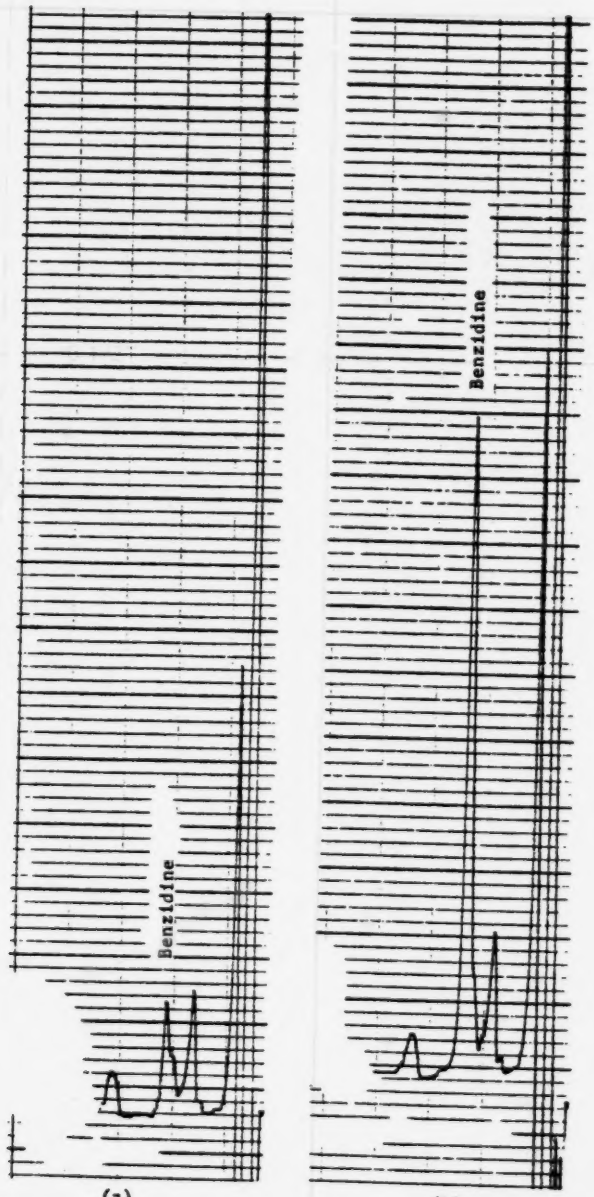


Figure 29. HPLC chromatogram of Sample 7 unspiked (a) and spiked with 50 ppb of benzidine and DCB (b) at 0.6 volts on a Spherisorb-ODS column.

31, respectively. Recoveries for benzidine and DCB spiked at the 10 ppb level were excellent both before and after storage for 48 hours at 4°C and pH 2. MDLs are approximately 1 ppb for both compounds, based on the sample background level.

As discussed in the Experimental Section preliminarily, two alternate approaches to benzidine analysis were evaluated, namely direct sample injection and resin adsorption. Figure 32 shows the result from the direct injection of an effluent from an organic chemical plant. It is readily seen that a few ppb of benzidine and DCB can be determined using this approach for this sample. While many samples are undoubtedly too complex to accomplish this by direct injection, this approach appears to be attractive for relatively clean waters since problems associated with concentrating large volumes of solvent are avoided.

The second approach, resin adsorption, is illustrated in Figure 33, wherein a secondary sewage sample (sample 2) is assayed unspiked and spiked at the 10 ppb level. This approach appears to be rather attractive and should be further investigated. However, it should be mentioned that a preliminary effort to assay dye plant effluents in this manner was not successful since large quantities of material were retained and overloaded the column.

An estimate of the precision and accuracy of the method can be obtained using the results for samples 2, 3, 4, 7, and 8 using Method 2. For these samples, the average recovery was 69 ± 15% for benzidine and 76 ± 9% for DCB. Samples 5 and 6 were omitted from this calculation because a degree of interference was suspected. The interference cannot be confirmed, however, since this was determined at only 0.8 volts and not at 0.6 volts, where more reliable results for benzidine are obtained.

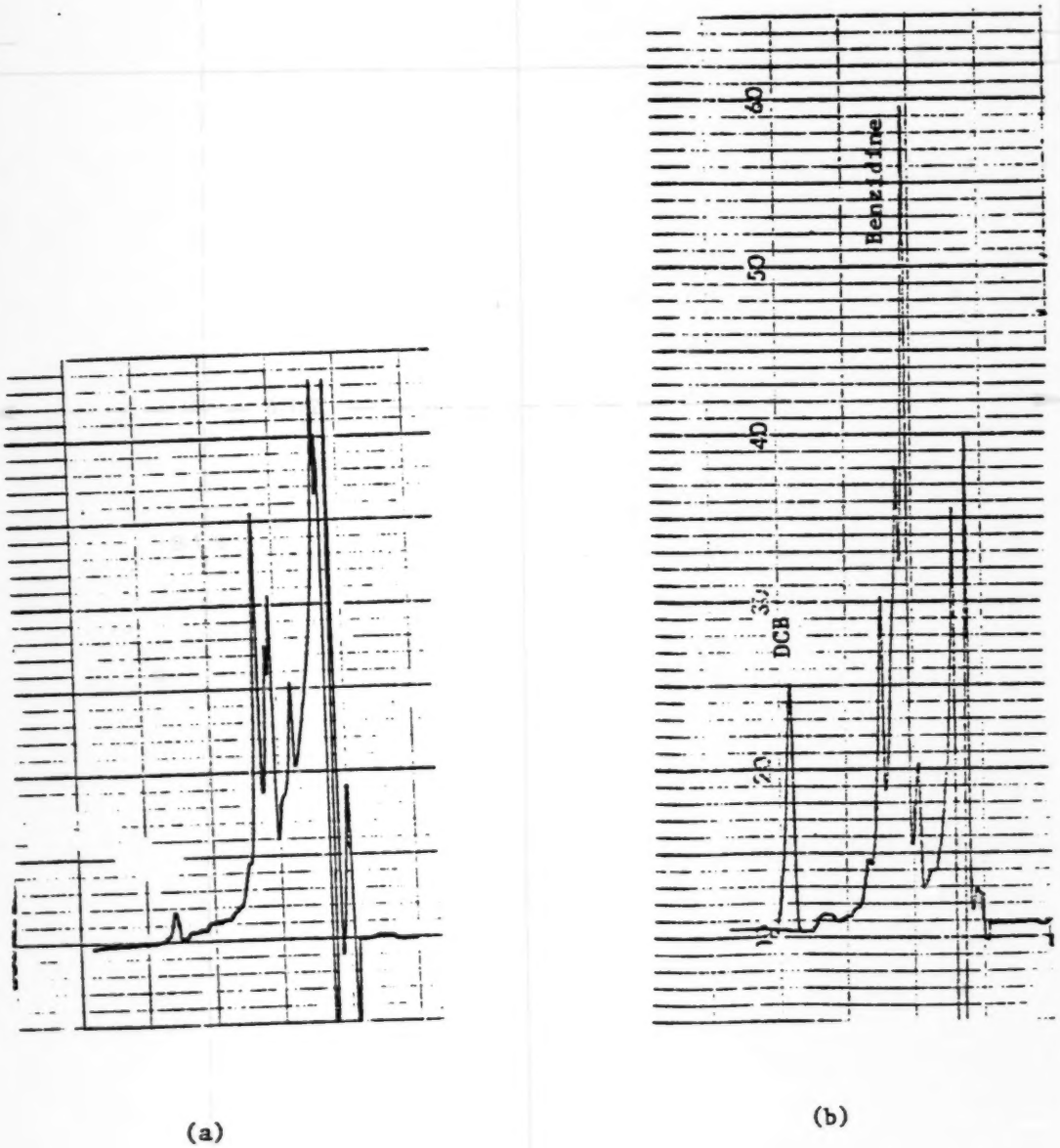
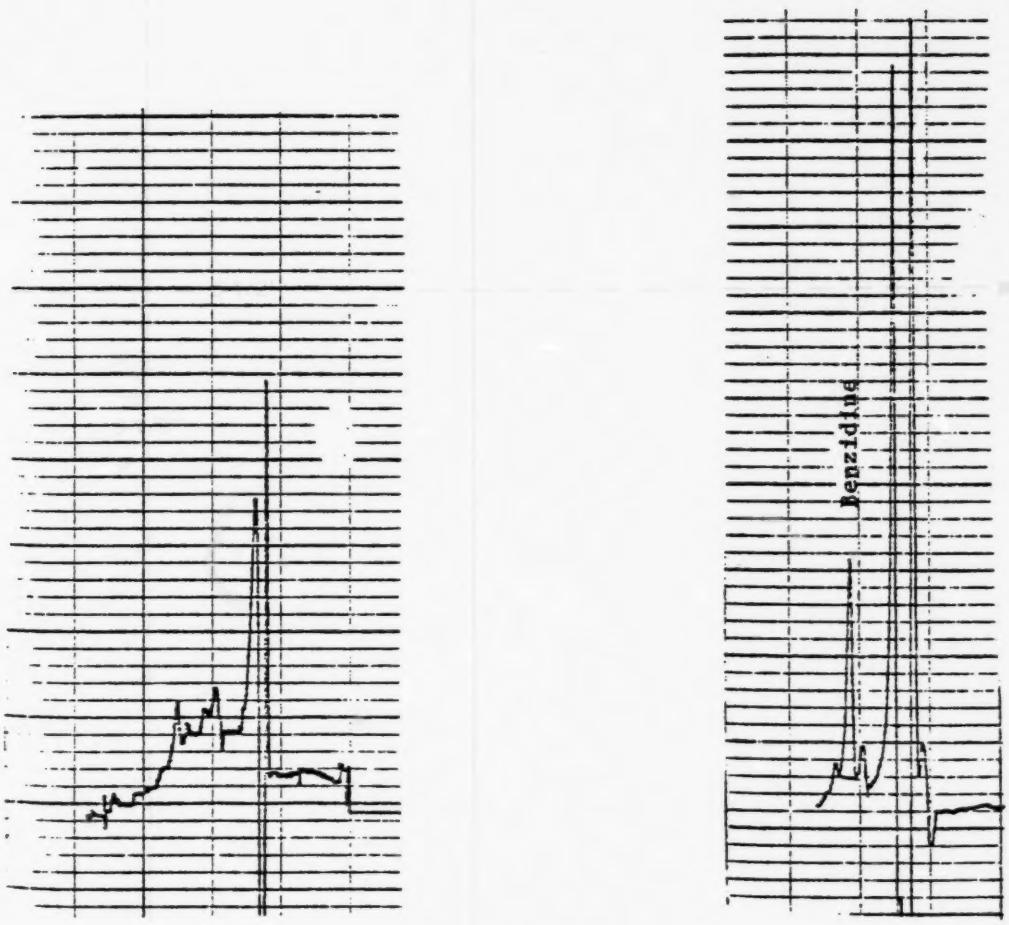


Figure 30. HPLC chromatograms for Sample 8 unspiked (a) and spiked with 10 ppb of benzidine at DCB (b) at 0.8 volts.



(a) (b)

Figure 31. HPLC chromatograms for Sample 8 unspiked (a) and spiked with 10 ppb of benzidine and DCB (b) at 0.6 volts.

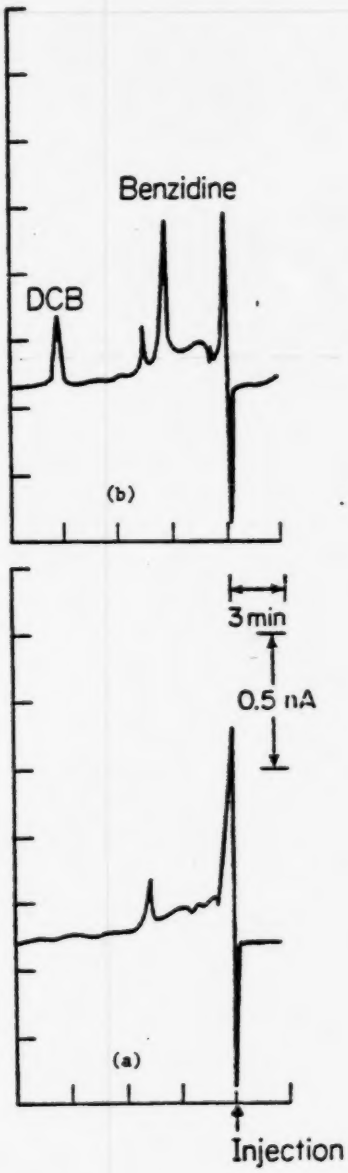


Figure 32. HPLC chromatogram for direct injection of a sample unspiked (a) and spiked with 10 ppb of benzidine and DCB (b).

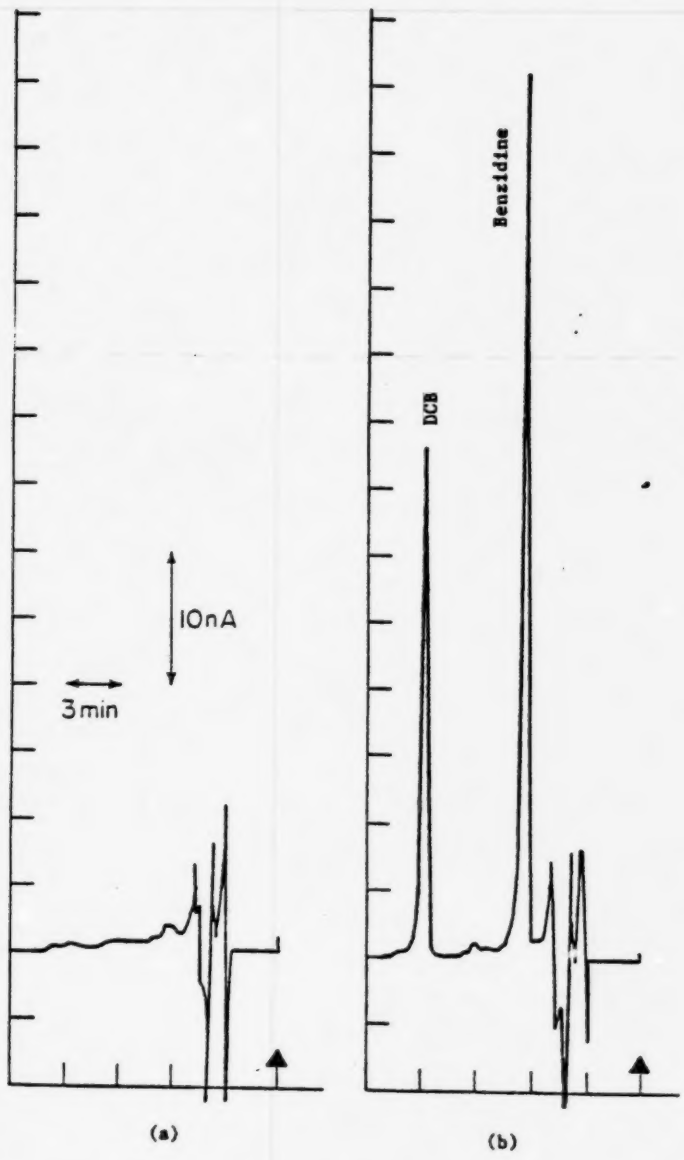


Figure 33. HPLC chromatogram for Sample 2 unspiked (a) and spiked with 10 ppb of benzidine and DCB (b) following resin adsorption.

SECTION 5

SUMMARY AND RECOMMENDATIONS

The conclusions drawn from this study are as follows:

1. DPH is too unstable in both organic solvents and aqueous solutions to permit meaningful analytical data to be obtained.

2. Benzidine and DCB are completely stable for at least 90 days in both methanol and acetonitrile when stored in the dark in sealed ampules.

3. Benzidine and DCB can be efficiently extracted from water with chloroform at pH 7 and pH 10. At pH 2, DCB is readily extracted but benzidine is not.

4. All three compounds are destroyed when stored in aqueous solutions containing 2 ppm of chlorine. In the absence of chloroform, benzidine and DCB are stable at pH 2 and pH 4.7. However, at elevated pH benzidine is more readily degraded, due to oxidative reactions.

5. Analysis of several wastewater samples showed that the analytical methods developed herein work well for the analysis of benzidine and DCB down to the 1 ppm level for most samples. Dye plant samples are a special case since those utilized in this program all showed apparently detectable 10-20 ppb levels of benzidine. Confirmed measurements on two HPLC columns indicate that benzidine is present at the levels stated, although more definitive measurements should be made.

The method presented in this work is believed to be an optimized method for the chromatographic determination of benzidine and DCB in aqueous effluents. It should be used for such analyses in a variety of laboratories to assure that it is suitable for routine monitoring purposes. Alternate approaches such as resin adsorption should be investigated further.

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5. Mondron, P. J. and P. G. Bonnett. The Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Abstract No. 437, 1978.

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APPENDIX

BENZIDINES

METHOD 605

1. Scope and Application

1.1 This method covers the determination of certain benzidines. The following parameters can be determined by this method:

Parameter	STORET No.	CAS No.
Benzidine	39120	92-87-5
3,3'-Dichlorobenzidine	34631	91-94-1

1.2 This is a high performance liquid chromatography (HPLC) method applicable to the determination of the compounds listed above in municipal and industrial discharges as provided under 40 CFR 136.1. When this method is used to analyze unfamiliar samples for the compounds above, identifications should be supported by, at least, one additional qualitative technique. This method describes electrochemical conditions at a second potential which can be used to confirm measurements made with this method. Method 625 provides gas chromatograph/mass spectrometer (GC/MS) conditions appropriate for the qualitative and quantitative confirmation of results of the parameters listed above, using the extract produced by this method.

1.3 The method detection limit (MDL defined in Section 14)[1] for each parameter is listed in Table 1. The MDL for a specific wastewater may differ depending upon the nature of the interferences in the sample matrix.

1.4 Any modification of this method, beyond those expressly permitted, shall be considered as major modifications subject to application and approval of alternate test procedures under 40 CFR 136.4 and 136.5.

1.5 This method is restricted to use by or under the supervision of analysts experienced in the use of HPLC instrumentation and in the interpretation of liquid chromatograms. Each analyst must demonstrate the ability to generate acceptable results with this method using the procedure described in Section 8.2.

2. Summary of Method

2.1 A measured volume of sample, approximately 1-liter, is extracted with chloroform using liquid-liquid extractions in a separatory funnel. The chloroform extract is back extracted with acid, neutralized, and extracted with chloroform. The chloroform is exchanged to methanol and concentrated using a rotary evaporator and nitrogen blowdown. It is then brought to a volume of 5 mL with an acetate buffer. HPLC conditions are described which permit the separation and measurement of the benzidine compounds using an electrochemical detector[2].

2.2 The acid back extraction step acts as a general-purpose cleanup to aid in the elimination of interferences.

3. Interferences

3.1 Method interferences may be caused by contaminants in solvents, reagents, glassware, and other sample processing hardware that lead to discrete artifacts and/or elevated baselines in chromatograms. All of these materials must be routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory reagent blanks as described in Section 8.5.

3.1.1 Glassware must be scrupulously cleaned[3]. Clean all glassware as soon as possible after use by rinsing with the last solvent used in it. This should be followed by detergent washing with hot water, and rinses with tap water and reagent water. It should then be drained dry, and heated in a muffle furnace at 400°C for 15 to 30 minutes. Some thermally stable materials, such as PCBs, may not be eliminated by this treatment. Solvent rinses with acetone and pesticide quality hexane may be substituted for the muffle furnace heating. Volumetric ware should not be heated in a muffle furnace. After drying and cooling, glassware should be sealed and stored in a clean environment to prevent any accumulation of dust or other contaminants. Store inverted or capped with aluminum foil.

3.1.2 The use of high purity reagents and solvents helps to minimize interference problems. Purification of solvents by distillation in all-glass systems may be required.

3.2 Matrix interferences may be caused by contaminants that are coextracted from the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the nature and diversity of the industrial complex or municipality being sampled. The cleanup procedures that are inherent in the extraction step are used to overcome many of these interferences, however unique samples may require additional clean-up approaches to achieve the MDL listed in Table 1.

3.3 Some dye plant effluents contain large amounts of components with retention times close to benzidine. In these cases, it has been found useful to reduce the electrode potential in order to eliminate interferences yet still detect benzidine. (See Section 12.7.)

4. Safety

4.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available and are identified [4-6] for the benefit of the analyst.

4.2 Benzidine, 3,3'-dichlorobenzidine and chloroform, used for extraction of samples have been tentatively classified as known or suspected, human or mammalian carcinogens.

5. Apparatus and Materials

5.1 Sampling equipment, for discrete or composite sampling.

5.1.1 Grab sample bottle - Amber glass, 1-liter or 1-quart volume, fitted with screw caps lined with Teflon. Foil may be substituted for Teflon if the sample is not corrosive. If amber bottles are not available, protect samples from light. The container must be washed, rinsed with acetone, or methylene chloride, and dried before use to minimize contamination.

5.1.2 Automatic sampler (optional) - Must incorporate glass sample containers for the collection of a minimum of 250 mL. Sample containers must be kept refrigerated at 4°C and protected from light during compositing. If the sampler uses a peristaltic pump, a minimum length of compressible silicone rubber tubing may be used. Before use, however, the compressible tubing should be thoroughly rinsed with methanol, followed by repeated rinsings with distilled water to minimize the potential for contamination of the sample. An integrating flow meter is required to collect flow proportional composites.

5.2 Glassware (All specifications are suggested.)

5.2.1 Separatory funnels - 2000, 1000, and 250-mL, with Teflon stopcock.

5.2.2 Vials - Amber glass, 10 to 15 mL capacity, with Teflon lined screw-cap.

5.2.3 Rotary evaporator.

5.2.4 Flasks, round bottom, 100-mL, with 24/40 joints.

5.2.5 Centrifuge tubes - conical, screw capped, graduated, equipped with Teflon lined caps.

5.2.6 Pipettes - Pasteur, with bulbs.

5.3 Balance - Analytical, capable of accurately weighing 0.0001 g.

5.4 High performance liquid chromatograph - An analytical system complete with column supplies, compatible recorder, high pressure syringes and the following components.

5.4.1 Solvent delivery system - with pulse damper, Altex 110A, or equivalent.

5.4.2 Injection valve (Optional) - Waters U6K or equivalent.

5.4.3 Electrochemical detector - Bioanalytical Systems LC-2A with glassy carbon electrode, or equivalent. This detector has proven effective in the analysis of wastewaters for the parameters listed in the scope, and was used to develop the method performance statements in Section 14. Guidelines for the use of alternate detectors are provided in Section 12.1.

5.4.4 Electrode polishing kit - Princeton Applied Research Model 9320 or equivalent.

5.4.5 Column - Lichrosorb RP-2, 5 micron particle diameter, in a 25 cm x 4.6 mm ID stainless steel column. This column was used to develop the method performance statements in Section 14. Guidelines for the use of alternate column packings are provided in Section 12.1.

6. Reagents

6.1 Reagent water - Reagent water is defined as a water in which an interferent is not observed at the method detection limit of each parameter of interest.

6.2 Sodium hydroxide solution (5 N) - (ACS) Dissolve 20g NaOH in reagent water and dilute to 100 mL.

6.3 Sodium hydroxide (1 M)-(ACS) - Dissolve 40 g NaOH in reagent water and dilute to one liter.

6.4 Sodium thiosulfate - (ACS) Granular.

6.5 Sodium tribasic phosphate (0.4 M) - (ACS) Dissolve 160 g of trisodium phosphate decahydrate in reagent water and dilute to one liter.

6.6 Sulfuric acid solution (1+1) - (ACS) Slowly, add 50 mL H_2SO_4 (sp. gr. 1.84) to 50 mL of reagent water.

6.7 Sulfuric acid (1 M) - (ACS) Slowly add 58 mL H_2SO_4 (sp. gr. 1.84) to reagent water and dilute to one liter.

6.8 Acetate buffer (0.1 M, pH-4.7) Dissolve 5.8 mL glacial acetic acid (ACS) and 13.6 grams of sodium acetate trihydrate (ACS) in reagent water which has been purified by filtration through a RO-4 Millipore System or equivalent and dilute to one liter.

6.9 Acetonitrile, chloroform (preserved with 1% ethanol), methanol - Pesticide quality or equivalent.

6.10 Mobile phase - Place equal volumes of filtered acetonitrile (Millipore type FH filter or equivalent) and filtered acetate buffer (Millipore type GS filter or equivalent) in a narrow mouth, glass container and mix thoroughly. Prepare fresh weekly. Degas daily by sonicating under vacuum, or by heating and stirring, or by purging with helium.

6.11 Stock standard solutions (1.00 µg/µL) - Stock standard solutions may be prepared from pure standard materials or purchased as certified solutions.

6.11.1 Prepare stock standard solutions by accurately weighing about 0.0100 grams of pure material. Dissolve the material in pesticide quality methanol and dilute to volume in a 10-mL volumetric flask. Larger volumes can be used at the convenience of the analyst. If compound purity is certified at 96% or greater, the weight can be used without correction to calculate the concentration of the stock standard. Commercially prepared stock standards can be used at any concentration if they are certified by the manufacturer or by an independent source.

6.11.2 Transfer the stock standard solutions into Teflon-sealed screw-cap bottles. Store at 4°C and protect from light. Stock standard solutions should be checked frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards from them. Quality control check samples that can be used to determine the accuracy of calibration standards will be available from the U. S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, in Cincinnati, Ohio.

6.11.3 Stock standard solutions must be replaced after six months, or sooner if comparison with quality control check samples

indicate a problem.

7. Calibration

7.1 Establish chromatographic operating parameters equivalent to those given in Table 1. The HPLC system can be calibrated using the external standard technique (Section 7.2) or the internal standard technique (Section 7.3).

7.2 External standard calibration procedure:

7.2.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask and diluting to volume with mobile phase. One of the external standards should be at a concentration near, but above, the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector.

7.2.2 Using syringe injections of 5 to 25 µL or a constant volume injection loop, inject each calibration standard, and tabulate peak height or area responses against the mass injected. The results can be used to prepare a calibration curve for each compound. Alternatively, if the ratio of response to amount injected (calibration factor) is a constant over the working range (< 10% relative standard deviation, RSD), linearity through the origin can be assumed and the average ratio or calibration factor can be used in place of a calibration curve.

7.2.3 The working calibration curve or calibration factor must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve or calibration factor must be prepared for that compound. If serious loss of sensitivity occurs, polish the electrode and recalibrate.

7.3 Internal standard calibration procedure. To use this approach, the analyst must select one or more internal standards that are similar in analytical behavior to the compounds of interest. The analyst must further demonstrate that the measurement of the internal standard is not affected by method or matrix interferences. Because of these limitations, no internal standard can be suggested that is applicable to all samples.

7.3.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask. To each calibration standard, add a known constant

amount of one or more internal standards, and dilute to volume with mobile phase. One of the standards should be at a concentration near, but above, the method detection limit and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector.

7.3.2 Using 5 to 25 µL aliquots or a constant volume injection loop, inject each calibration standard, and tabulate peak height or area responses against concentration for each compound and internal standard, and calculate response factors (RF) for each compound using equation 1.

$$\text{Eq. 1} \quad RF = (A_s C_{is})/(A_{is}\ C_s)$$

where:

A_s = Response for the parameter to be measured.
A_{is} = Response for the internal standard.
C_{is} = Concentration of the internal standard, (µg/L).
C_s = Concentration of the parameter to be measured, (µg/L).

If the RF value over the working range is a constant (< 10% RSD), the RF can be assumed to be nonvariant and the average RF can be used for calculations. Alternatively, the results can be used to plot a calibration curve of response ratios, A_s/A_{is}, vs. RF.

7.3.3 The working calibration curve or RF must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve must be prepared for that compound. If serious loss of response occurs, polish the electrode and recalibrate.

7.4 Before using any alternate cleanup procedure, the analyst must process a series of calibration standards through the procedure to validate elution patterns and the absence of interferences from the reagents.

8. Quality Control

8.1 Each laboratory that uses this method is required to operate a formal quality control program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the analysis of spiked samples as a continuing check on performance. The laboratory is required to maintain performance records to define the quality of data that is generated. After January 1, 1982, ongoing performance checks must be compared with

established performance criteria to determine if the results of analyses are within accuracy and precision limits expected of the method.

8.1.1 Before performing any analyses, the analyst must demonstrate the ability to generate acceptable accuracy and precision with this method. This ability is established as described in Section 8.2.

8.1.2 In recognition of the rapid advances that are occurring in chromatography, the analyst is permitted certain options to improve the separations or lower the cost of measurements. Each time such modifications are made to the method, the analyst is required to repeat the procedure in Section 8.2.

8.1.3 The laboratory must spike and analyze a minimum of 10% of all samples to monitor continuing laboratory performance. This procedure is described in Section 8.4.

8.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations.

8.2.1 Select a representative spike concentration for each compound to be measured. Using stock standards, prepare a quality control check sample concentrate in methanol 1000 times more concentrated than the selected concentrations. Quality control check sample concentrates, appropriate for use with this method, will be available from the U. S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

8.2.2 Using a pipet, add 1.00 mL of the check sample concentrate to each of a minimum of four 1000-mL aliquots of reagent water. A representative wastewater may be used in place of the reagent water, but one or more additional aliquots must be analyzed to determine background levels, and the spike level must exceed twice the background level for the test to be valid. Analyze the aliquots according to the method beginning in Section 10.

8.2.3 Calculate the average percent recovery, (R), and the standard deviation of the percent recovery (s), for the results. Wastewater background corrections must be made before R and s calculations are performed.

8.2.4 Using the appropriate data from Table 2, determine the recovery and single operator precision expected for the method, and compare these results to the values measured in Section 8.2.3. If the data are not comparable, the analyst must review potential problem areas and repeat the test.

8.2.5 After January 1, 1982, the values for R and s must meet rigid method performance criteria provided by the U.S. EPA, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio, before any samples may be analyzed.

8.3 The analyst must calculate method performance criteria and define the performance of the laboratory for each spike concentration and parameter being measured.

8.3.1 Calculate upper and lower control limits for method performance:

$$\text{Upper Control Limit (UCL)} = R + 3s$$
$$\text{Lower Control Limit (LCL)} = R - 3s$$

where R and s are calculated as in Section 8.2.3. The UCL and LCL can be used to construct control charts[7] that are useful in observing trends in performance. After January 1, 1982, the control limits above must be replaced by method performance criteria provided by the U.S. Environmental Protection Agency.

8.3.2 The laboratory must develop and maintain separate accuracy statements of laboratory performance for wastewater samples. An accuracy statement for the method is defined as $R \pm s$. The accuracy statement should be developed by the analysis of four aliquots of wastewater as described in Section 8.2.2, followed by the calculation of R and s. Alternately, the analyst may use four wastewater data points gathered through the requirement for continuing quality control in Section 8.4. The accuracy statements should be updated regularly[7].

8.4 The laboratory is required to collect a portion of their samples in duplicate to monitor spike recoveries. The frequency of spiked sample analysis must be at least 10% of all samples or one sample per month, whichever is greater. One aliquot of the sample must be spiked and analyzed as described in Section 8.2. If the recovery for a particular parameter does not fall within the control limits for method performance, the results reported for that parameter in all samples processed as part of the same set must be qualified as described in Section 13.3. The laboratory should monitor the frequency of data so qualified to ensure that it remains at or below 5%.

8.5 Before processing any samples, the analyst should demonstrate through the analysis of a one-liter aliquot of reagent water, that all glassware and reagents interferences are under control. Each time a set of samples is extracted or there is a change in reagents, a laboratory reagent blank should be processed as a safeguard against laboratory contamination.

8.6 It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Field duplicates may be analyzed to monitor the precision of the sampling technique. When doubt exists over the identification of a peak on the chromatogram, confirmatory techniques such as HPLC with a dissimilar column, gas chromatography, or mass spectrometry must be used. Whenever possible, the laboratory should perform analysis of standard reference materials and participate in relevant performance evaluation studies.

9. Sample Collection, Preservation, and Handling

9.1 Grab samples must be collected in glass containers. Conventional sampling practices [8] should be followed, except that the bottle must not be prewashed with sample before collection. Composite samples should be collected in refrigerated glass containers in accordance with the requirements of the program. Automatic sampling equipment must be as free as possible of Tygon and other potential sources of contamination.

9.2 The samples must be iced or refrigerated at 4°C and stored in the dark from the time of collection until extraction. Both benzidine and 3,3'-dichlorobenzidine are easily oxidized. Fill the sample bottle and, at time of collection, if residual chlorine is present, add 80 mg of sodium thiosulfate per liter of sample, and mix thoroughly. EPA Methods 330.4 and 330.5 may be used for measurement of residual chlorine [9]. Field test kits are available for this purpose. After mixing, adjust the pH of the sample to a range of 2 to 7 with sulfuric acid.

9.3 If 1,2-diphenyl hydrazine is likely to be present, adjust the pH of the sample to 4.0 ± 0.2 to prevent rearrangement to benzidine.

9.4 All samples must be extracted within 7 days. Extracts may be held up to 7 days before analysis, if stored under an inert (oxidant free) atmosphere [2]. The extract should be protected from light.

10. Sample Extraction

10.1 Mark the water meniscus on the side of the sample bottle for later determination of sample volume. Pour the entire sample into a two-liter separatory funnel. Check the pH of the sample with wide-range pH paper and adjust to within the range of 6.5 to 7.5 with sodium hydroxide or sulfuric acid solutions.

10.2 Add 100 mL chloroform to the sample bottle, seal, and shake 30 seconds to rinse the inner walls. (Caution: Handle chloroform in a well ventillated area.) Transfer the solvent to the separatory funnel and extract the sample by shaking the funnel for two minutes with periodic venting to release excess pressure. Allow the organic

layer to separate from the water phase for a minimum of ten minutes. If the emulsion interface between layers is more than one-third the volume of the solvent layer, the analyst must employ mechanical techniques to complete the phase separation. The optimum technique depends upon the sample, but may include stirring, filtration of the emulsion through glass wool, centrifugation, or other physical methods. Collect the chloroform extract in a 250-mL separatory funnel.

10.3 Add a 50-mL volume of chloroform to the sample bottle and repeat the extraction procedure a second time, combining the extracts in the separatory funnel. Perform a third extraction in the same manner.

10.4 Separate and discard any aqueous layer remaining in the 250-mL separatory funnel after combining the organic extracts. Add 25 mL of 1 M sulfuric acid and extract the sample by shaking the funnel for two minutes. Transfer the aqueous layer to a 250-mL beaker. Extract with two additional 25-mL portions of 1 M sulfuric acid and combine the acid extracts in the beaker.

10.5 Place a stirbar in the 250-mL beaker and stir the acid extract while carefully adding 5 mL of 0.4 M sodium tribasic phosphate. While monitoring with a pH meter, neutralize the extract to a pH between 6 and 7 by dropwise addition of 5 N NaOH while stirring the solution vigorously. Approximately 25 to 30 mL of 5 N NaOH will be required and it should be added over at least a 2-minute period. Do not allow the sample pH to exceed 8.

10.6 Transfer the neutralized extract into a 250-mL separatory funnel. Add 30 mL of chloroform and shake the funnel for 2 minutes. Allow the phases to separate, and transfer the organic layer to a second 250-mL separatory funnel.

10.7 Extract the aqueous layer with two additional 20-mL aliquots of chloroform as before. Combine the extracts in the 250-mL separatory funnel.

10.8 Add 20 mL of reagent water to the combined organic layers and shake for 30 seconds.

10.9 Transfer the organic extract into a 100-mL round bottom flask. Add 20 mL of methanol and concentrate to 5 mL with a rotary evaporator at reduced pressure and 35°C. An aspirator is recommended for use as the source of vacuum. Chill the receiver with ice. This operation requires approximately 10 minutes. Other concentration techniques may be employed, if the requirements of Section 8.2 are met.

10.10 Using a 9-inch Pasteur pipette, transfer the extract to a 15 mL conical screw-capped centrifuge tube. Rinse the flask, including the entire side wall, with 2-mL portions of methanol

and combine with the original extract.

10.11 Carefully concentrate the extract to 0.5 mL using a gentle stream of nitrogen while heating in a 30°C water bath. Dilute to 2 mL with methanol, reconcentrate to 1 mL, and dilute to 5 mL with acetate buffer. Mix extract thoroughly. Cap the centrifuge tube and store refrigerated and protected from light if further proces- sing will not be performed immediately.

10.12 Determine the original sample volume by refilling the sample bottle to the mark and transferring the liquid to a 1000 mL graduated cylinder. Record the sample volume to the nearest 5 mL.

11. Cleanup and Separation

11.1 Additional cleanup procedures may not be necessary for a relatively clean sample matrix. The single operator precision and accuracy data in Section 14 were gathered using only those cleanup procedures that are inherent in the extraction procedures of this method. If particular circumstances demand the use of an alternative cleanup procedure, the analyst must determine the elution profile and demonstrate that the recovery of each compound of interest is no less than 85%.

12. Liquid Chromatography

12.1 Table 1 summarizes the recommended operating conditions for the HPLC. This Table includes retention times, capacity factors, and method detection limits that were obtained under these conditions. An example of the parameter separation achieved by the HPLC column is shown in Figure 1 . Other HPLC columns, chromatographic conditions, or detectors may be used if the requirements of Section 8.2 are met. When the HPLC is idle, it is advisable to maintain a 0.1 mL/min flow through the column to prolong column life.

12.2 Calibrate the system daily as described in Section 7.

12.3 If the internal standard approach is being used, the standard must be added to the sample extract, and mixed thoroughly immediately, before injection into the instrument.

12.4 Inject 5 to 25 µL of the sample extract. If constant volume injection loops are not used, record the volume injected to the nearest 0.05 µL, and the resulting peak size in area or peak height units.

12.5 The width of the retention time window used to make identifications should be based upon measurements of actual retention time variations of standards over the course of a day. Three times the standard deviation of a retention time for a compound can be used

to calculate a suggested window size; however, the experience of the analyst should weigh heavily in the interpretation of chromatograms.

12.6 If the response for the peak exceeds the working range of the system, dilute the extract with mobile phase and reanalyze.

12.7 If the measurement of the peak response for benzidine is prevented by the presence of interferences, reduce the electrode potential to +0.6 volts and reanalyze. If the benzidine peak is still obscured by interferences, further cleanup is required.

13. Calculations

13.1 Determine the concentration of individual compounds in the sample.

13.1.1 If the external standard calibration procedure is used, calculate the amount of material injected from the peak response using the calibration curve or calibration factor in Section 7.2.2. The concentration in the sample can be calculated from equation 2:

$$\text{Eq. 2.} \quad \text{Concentration, } \mu g/L = \frac{(A)(V_t)}{(V_i)(V_s)}$$

where:

A = Amount of material injected, in nanograms.
V_i = Volume of extract injected (µL).
V_t = Volume of total extract (µL).
V_s = Volume of water extracted (mL).

13.1.2 If the internal standard calibration procedure was used, calculate the concentration in the sample using the response factor (RF) determined in Section 7.3.2 and equation 3.

$$\text{Eq. 3.} \quad \text{Concentration, } \mu g/L = \frac{(A_s)\ (I_s)}{(A_{is})(RF)(V_o)}$$

where:

A_s = Response for the parameter to be measured.
A_{is} = Response for the internal standard.
I_s = Amount of internal standard added to each extract (µg).
V_o = Volume of water extracted, in liters.

13.2 Report results in micrograms per liter without correction for recovery data. When duplicate and spiked samples are analyzed, report all data obtained with the sample results.

13.3 For samples processed as part of a set where the laboratory spiked sample recovery falls outside of the control limits in Section 8.4, data for the affected parameters must be labeled as suspect.

14. Method Performance

14.1 The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero[1]. The MDL concentrations listed in Table 1 were obtained using reagent water[10]. Similar results were achieved using representative wastewaters.

14.2 This method has been tested for linearity of analyte recovery from reagent water and has been demonstrated to be applicable over the concentration range from 7 x MDL to 3000 x MDL[10].

14.3 In a single laboratory (Battelle, Columbus Laboratories), using spiked wastewater samples, the average recoveries presented in Table 2 were obtained[2]. Each spiked sample was analyzed in triplicate on two separate days. The standard deviation of the percent recovery is also included in Table 2.

14.4 The Environmental Protection Agency is in the process of conducting an interlaboratory method study to fully define the performance of this method.

References

1. "Methods for Organic Chemical Analysis of Water and Wastes by GC, HPLC, and GC/MS," U. S. EPA, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268, July 1981.

2. "Determination of Benzidines in Industrial and Municipal Wastewaters," Report for EPA Contract 68-03-2624 (In preparation).

3. ASTM Annual Book of Standards, Part 31, D3694, "Standard Practice for Preparation of Sample Containers and for Preservation," American Society for Testing and Materials, Philadelphia, PA, p. 679, 1980.

4. "Carcinogens - Working With Carcinogens," Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health, Publication No. 77-206, Aug. 1977.

5. "OSHA Safety and Health Standards, General Industry," (29 CFR 1910), Occupational Safety and Health Administration, OSHA 2206, (Revised, January 1976).

6. "Safety in Academic Chemistry Laboratories," American Chemical Society Publication, Committee on Chemical Safety, 3rd Edition, 1979.

7. "Handbook for Analytical Quality Control in Water and Wastewater Laboratories," EPA-600/4-79-019, U. S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory - Cincinnati, Ohio 45268, March 1979.

8. ASTM Annual Book of Standards, Part 31, D3370, "Standard Practice for Sampling Water," American Society for Testing and Materials, Philadelphia, PA, p. 76, 1980.

9. "Methods 330.4 (Titrimetric, DPD-FAS) and 330.5 (Spectrophotometric, DPD) for Chlorine, Total Residual," Methods for Chemical Analysis of Water and Wastes, EPA 600/4-79-020, U. S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory - Cincinnati, Ohio 45268, March 1979.

10. "Determination of Method Detection Limit and Analytical Curve for EPA Method 605 - Benzidines," Special letter report for EPA Contract 68-03-2624, Environmental Monitoring and Support Laboratory - Cincinnati, Ohio 45268.

TABLE 1

CHROMATOGRAPHIC CONDITIONS AND METHOD DETECTION LIMITS

Parameter	Retention Time (min)	Column Capacity Factor	Method Detection Limit (µg/L)
Benzidine	6.1	1.44	0.08
3,3'-dichlorobenzidine	12.1	3.84	0.13

HPLC Column conditions: Lichrosorb RP-2, 5 micron particle size, in a 25 cm x 4.6 mm ID stainless steel column. Mobile Phase: 0.8 mL/min of 50% acetonitrile/50% 0.1M pH 4.7 acetate buffer. The method detection limit was determined using an electrochemical detector operated at + 0.8 volts.

TABLE 2

SINGLE OPERATOR ACCURACY AND PRECISION

Parameter	Average Percent Recovery	Standard Deviation %	Spike Range (µg/L)	Number of Analyses	Matrix Types
Benzidine	65	11.4	1.0-50	30	5
3,3'-Dichlorobenzidine	64	9.6	1.0-50	30	5

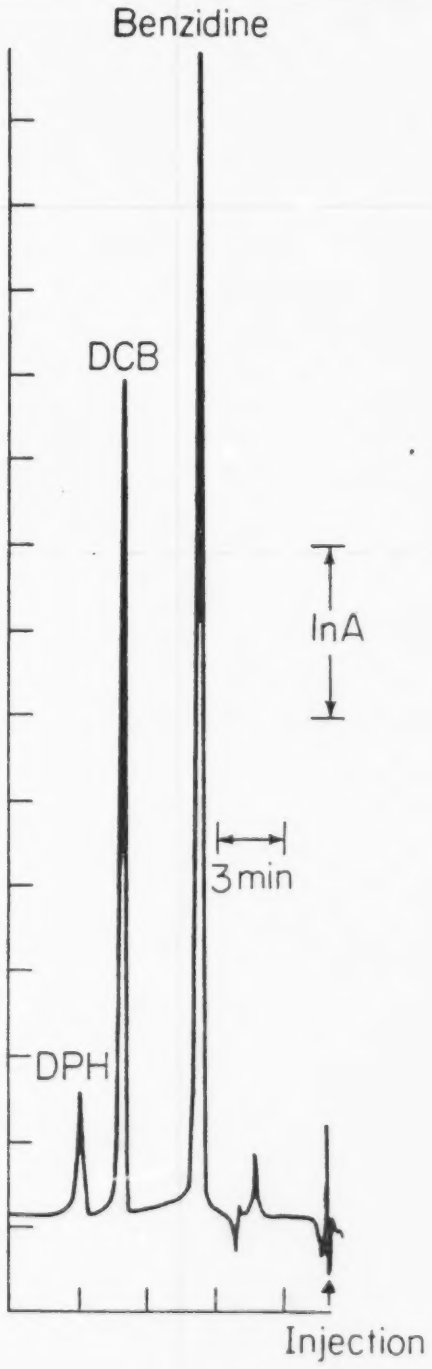

Figure 1. HPLC chromatogram of Benzidines